HERBS, SPICES, VEGETABLES AND BERRIES: ANTIOXIDANT-RELATED ACTIVITIES

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Tiivistelmä – Referat – Abstract

Numerous scientific studies have revealed the connection between oxidative stress and a wide range of diseases, including cardiovascular, neurodegenerative, inflammatory diseases and cancer. The most probable theory of ageing is based on oxidative stress as well. There exist endogenous and exogenous antioxidants capable of fighting oxidative and nitrosative damage to molecules and tissues of the body. Such compounds may be beneficial in prevention and treatment of different conditions. For example, plant foods contain various amounts of antioxidants.

The aim of this thesis is to evaluate the antioxidant-related activities of certain commonly used vegetables (broccoli, brussel sprouts, cauliflower, peas), berries (bilberry, raspberry), herbs (Egyptian basil, oregano, rosemary, thyme) and spices (paprika) and to discuss their role in human health.

The sample extracts were tested with four different methods: the determination of total phenols using Folin-Ciocalteu reagent, the DPPH free radical scavenging activity assay, reducing power activity assay and iron (II) chelation. In all assays, with exception of iron (II) chelation, vegetables proved to be less active as a potential source of antioxidants than other samples, while herbs seemed to be the most active samples. Iron chelation potential of samples is approximately the same with exception of paprika (lower than other samples) and bilberry (higher than other samples). The results obtained from different assays are not consistent with each other, and good correlative relationship occurs only between total phenols and iron reduction.

On the basis of the results, it can be assumed that herbs and berries may be the main target for the research of pharmaceutically important antioxidants, although in daily diet vegetables and fruits are likely to be the best sources of such compounds. However, the beneficial daily doses of plant foods remain to be considered and further research is needed to provide information on the activity of given samples *in vivo*.

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Antioxidant, culinary plants, total phenols, DPPH, reducing power, iron chelation

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Tiivistelmä – Referat – Abstract

Useat tieteelliset tutkimukset ovat osoittaneet yhteyden oksidatiivisen stressin ja erilaisten sairauksien, mukaan lukien sydän- ja verisuoni-, hermostoa rappeuttavien, tulehduksellisten ja syöpäsairauksien, välillä. Ikääntymisen todennäköisin teoria myös perustuu oksidatiiviseen stressiin. On olemassa endogeenisia ja eksogeenisia antioksidantteja, jotka kykenevät taistelemaan elimistön molekyyleille ja kudoksille aiheuttamia oksidatiivisia ja nitrosatiivisia vaurioita vastaan. Tällaiset yhdisteet voivat olla hyödyllisiä erilaisten sairauksien ehkäisyssä ja hoidossa. Esimerkiksi ruokakasvit sisältävät vaihtelevia määriä antioksidantteja.

Tämän työn tarkoituksena on määrittää tiettyjen yleisten vihannesten (parsakaali, ruusukaali, kukkakaali, herneet), marjojen (metsämustikka, vadelma), yrttien (egyptiläinen basilika, oregano, rosmariini, timjami) ja mausteiden (paprika) antioksidanttiaktiivisuuksia sekä keskustella niiden roolista ihmisterveydelle.

Näyteuutteet testattiin neljällä eri menetelmällä, joita ovat kokonaisfenolien määritys Folin-Ciocalteu reagenssia käyttäen, DPPH -vapaan radikaalin sieppaukseen perustuva määritys, pelkistyskykymääritys sekä raudan (II) kelaatio. Kaikissa määrityksissä, raudan (II) kelaatiota lukuun ottamatta, vihannekset osoittautuivat vähemmän aktiivisiksi mahdollisiksi antioksidanttilähteiksi kuin muut näytteet, kun taas yrtit näyttivät olevan aktiivisimpia näytteitä. Raudan kelaatiopotentiaali on suunnilleen sama kaikilla näytteillä, lukuun ottamatta paprikaa (pienempi kuin muilla näytteillä) ja metsämustikkaa (suurempi kuin muilla näytteillä). Eri määrityksistä saadut tulokset eivät vastaa toisiaan, ja hyvä korrelaatiosuhde on saatu ainoastaan kokonaisfenolien ja raudanpelkistyksen välillä.

Tulosten perusteella voidaan olettaa, että yrtit ja marjat voivat olla pääkohteita farmaseuttisesti tärkeiden antioksidanttien tutkimuksessa, vaikka päivittäisessä ruokavaliossa vihannekset ja hedelmät ovat todennäköisesti parhaita antioksidanttien lähteitä. Kuitenkin ruokakasvien hyödylliset päiväannokset ovat vielä selvittämättä ja lisätutkimuksia tarvitaan antamaan tietoa kyseessä olevien näytteiden aktiivisuuksista *in vivo*.

Avainsanat – Nyckelord – Keywords

Antioksidantti, ruokakasvit, kokonaisfenolit, DPPH, pelkistyskyky, raudan kelaatio

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I. LITERATURE

1. INTRODUCTION

The interest towards dietary antioxidants has arisen from the observation that the incidence of coronary heart diseases among French people remains low despite of their relatively high consumption of saturated fats and alcohol, especially red wine, which is commonly known as the ”French paradox” (Artaud-Wild et al., 1993). A possible explanation could be found in the greater consumption of plant-derived foods compared with the countries with higher rates of the coronary heart disease mortality. Fruits, vegetables and other plant foods widely consumed in Mediterranean diets contain a high percentage of health promoting and protective compounds known as antioxidants.

According to Halliwell and Gutteridge (2007) an antioxidant is “any substance that delays, prevents or removes oxidative damage to a target molecule”. The damage to DNA, lipids or proteins is usually a consequence of the action of free radicals. Free radicals can be determined as “any species capable of independent existence that contains one or more unpaired electrons” (Halliwell and Gutteridge, 2007). This definition is not the only one, but it is the most relevant to this thesis. There are also non-radical reactive species, which exist in a molecular state but can react with other compounds to produce radicals.

Numerous scientific studies have revealed the connection between oxidative stress caused by free radicals and a wide range of diseases, including cardiovascular, neurodegenerative, inflammatory diseases and cancer, amongst others (Dreher and Junod, 1996; Halliwell, 2001; Halliwell and Gutteridge, 2007). Thus, it has been suggested that a diet rich in antioxidants may retard the onset of such diseases or improve the symptoms.

The purpose of this thesis is to evaluate the antioxidant-related properties of some commonly used vegetables, berries, herbs and spices and to discuss their role in human health. *In vitro* assays applied in this research can give valuable information on the

potential benefits of tested samples in scavenging reactive species and preventing the initiation of oxidation chain reactions. The results cannot be directly interpreted in terms of *in vivo* efficacy, but they can provide the direction for further research.

2. OXIDATION AND OXIDATIVE STRESS

Molecular oxygen is a molecule composed of two oxygen atoms and it forms an important part of the atmosphere. Oxygen is vital for most living organisms, but it is also a source of endogenous oxidants. Various reactive species, which are either radicals or non-radicals capable of producing radical species, are formed during normal metabolic processes. Reactive oxygen species (ROS) include superoxide, hydroperoxyl, hydroxyl, alkylperoxyl, alkoxyl, carbonate and carbon dioxide radicals, while hydrogen peroxide and ozone represent non-radical species (Table 1) (Halliwell and Gutteridge, 2007). Nitrogen reactive species (RNS) can be divided into radicals and non-radicals as well (Table 1).

Sufficiently high oxygen concentrations can damage tissues increasing the incidence of tumours and other injuries (Plaine, 1955). Oxygen can cause auto-oxidation of oxygen sensitive compounds. Auto-oxidation may activate DNA damaging species, such as polycyclic aromatic hydrocarbons, and induce the production of hydrogen peroxide and other ROS (Lorentzen and Ts'o, 1977). The auto-oxidation of fats is called lipid peroxidation (Burton and Ingold, 1986).

Free radical reactions usually take place as a chain reaction consisting of initiation, propagation and termination steps (Figure 1) (Burton and Ingold, 1986). The initiation step comprises the production of a radical. In the propagation multi-step reactions a radical reacts with non-radical compounds to produce new reactive species. Those chain reactions continue until two radicals react with each other to produce a non-reactive molecular product or an antioxidant breaks the chain through reaction with a radical. This step is called termination.

Table 1. The examples of different reactive species (Modified from Halliwell and Gutteridge, 2007).

Radicals	**Non-radicals**
Reactive oxygen species	
Singlet oxygen ($^1O_2$)	Singlet oxygen ($^1O_2$)
Superoxide ($O_2^{\bullet -}$)	Hydrogen peroxide (H_2O_2)
Hydroperoxyl ($HO_2^{\bullet}$)	Organic peroxides (ROOH)
Hydroxyl ($OH^{\bullet}$)	Peroxomonocarbonate ($HOOCO_2^-$)
Peroxyl ($RO_2^{\bullet}$)	Ozone (O_3)
Alkoxyl ($RO^{\bullet}$)	Nitrosoperoxycarbonate ($ONOOCO_2^-$)
Carbonate ($CO_3^{\bullet -}$)	
Carbon dioxide ($CO_2^{\bullet -}$)	
Reactive nitrogen species	
Nitric oxide ($NO^{\bullet}$)	Nitrous acid (HNO_2)
Nitrogen dioxide ($NO_2^{\bullet}$)	Peroxynitrate (O_2NOO^-)
Nitrate ($NO_3^{\bullet}$)	Peroxynitrite ($ONOO^-$)
	Peroxynitrous acid (ONOOH)
	Peroxyacetyl nitrate [$CH_3C(O)OONO_2$]
	Nitrosyl cation (NO^+)
	Nitroxyl anion (NO^-)
Others	
Atomic chlorine ($Cl^{\bullet}$)	Hypochlorous acid (HOCl)
Atomic bromine ($Br^{\bullet}$)	Chloramines (R_2NCl)
	Chlorine gas (Cl_2)
	Chlorine dioxide (ClO_2)
	Bromine chloride (BrCl)
	Hypobromous acid (HOBr)
	Bromine gas (Br_2)

Initiation:	$RH \rightarrow R^{\bullet}$
Propagation:	$R^{\bullet} + O_2 \rightarrow ROO^{\bullet}$
	$ROO^{\bullet} + RH \rightarrow ROOH + R^{\bullet}$
Termination:	$ROO^{\bullet} + ROO^{\bullet} \rightarrow$ molecular products

Figure 1. The steps of free radical reaction chain (Modified from Burton and Ingold, 1986). RH = substrate molecule, $^{\bullet}$ = single electron indicating the radical

Iron and copper are capable of catalyzing free radical reactions (Halliwell and Gutteridge, 1984). Thus excessive free iron and/or copper ions can promote free radical formation and hence cytotoxicity in live tissues.

Exogenous sources of oxidants include various air pollutants, cigarette smoke, ionizing radiation and exposure to heavy metals. The organism can also receive oxidative compounds with food which components either are oxidants themselves or produce oxidants in the body through redox-type reactions.

There exist natural antioxidant defence mechanisms protecting the organism from harmful actions of oxidants which are produced during normal cell and tissue functions. The organism is able to synthesize proteins and small molecules which act as endogenous antioxidants. Those are specific enzymes such as superoxide dismutases, catalases and peroxidases (Brawn and Fridovich, 1981; Brigelius-Flohé, 1999). Uric acid which exists at relatively high concentrations in human plasma seems to be a powerful endogenous free radical scavenger as well (Ames et al., 1981). There exist also DNA repair mechanisms, glycosylases, that may eliminate the lesions already caused by oxidation (Hollstein et al., 1984).

The most important exogenous antioxidants involved in natural antioxidant defence are ascorbate (vitamin C), α-tocopherol (vitamin E) and selenium (Burton and Ingold, 1986; Frei et al., 1988; Brigelius-Flohé, 1999). Antioxidants usually cooperate with each other to provide better protection against oxidation than any of them could provide alone (Burton and Ingold, 1986; Fiskin et al., 2006) For example, vitamin E requires ascorbic

acid to recycle it *in vivo* (Burton and Ingold, 1986).

Oxidative stress occurs in situations, when natural antioxidant defence of the human body is not sufficient to fight excessive generation of reactive species (Halliwell, 2001). Cells may overcome oxidative stress by raising their antioxidant defence and/or repair capacity to minimize the consequences, but in some cases, oxidative damage occurs. Damaged biomolecules and cells in turn induce a range of diseases. At least cancer, cardiovascular and neurodegenerative diseases are associated with oxidative stress (Dreher and Junod, 1996; Halliwell, 2001; Halliwell and Gutteridge, 2007). Such diseases are often strongly associated with ageing, and there is evidence that oxidative damage to DNA, lipids and proteins increases in age-dependent manner (Mecocci et al., 1999; Hamilton et al., 2001; Venkateshappa et al., 2012).

2.1. The role of oxidative stress in ageing

The most probable theory of ageing is based on the oxidative stress damaging DNA and other macromolecules. Experiments on mice show that the sensitivity of DNA to oxidative damage increases with age, although the activities of the major antioxidant enzymes remain the same as in younger individuals (Hamilton et al., 2001). According to these experiments, at least in rodents age-related increase in DNA oxidation is greatest in brain and heart tissues, which could explain the rise of neurodegenerative and cardiovascular diseases with age. The increasing oxidative damage to DNA at least partially explains growing cancer incidence as well (Ames, 1989). There is also evidence from human studies that oxidation of DNA, lipids and proteins takes place in ageing skeletal muscle thus affecting its maximal functional activity (Mecocci et al., 1999).

It is not easy to determine exactly how profound role oxidative stress plays in ageing, as well as in other conditions. For example, in cohort study on skin ageing oxidative DNA damage was suggested to be one of the factors affecting the appearance of signs of ageing (Allerhand et al., 2011). The other factors were sex, body mass index and social class. Noteworthy, there seemed to be no correlation between DNA damage and

wrinkles, but pigmented spots and sagging were associated with oxidative damage. However, since the oxidative damage was evaluated only on basis of the concentration of DNA damage biomarker, it is possible, that there is an association between ageing and oxidation of other biomolecules.

2.2. The role of oxidative stress in the development of cancer

Cancer can develop from damage to DNA, which results in uncontrollable cell growth and/or the inhibition of apoptosis. As reactive species can damage DNA in many different ways, it is more than possible that oxidative stress plays an important part in the development of cancer. Research on the effects of reactive species has revealed plenty of possible mechanisms behind the induction of mutagenesis (Table 2). Lipid peroxidation can also play an important role in the development of certain types of cancer, such as breast cancer (Boyd and McGuire, 1991). Due to oxidative stress, at least some tumours may enhance their malignant potency through the activation of vascular endothelial growth factor and, as a consequence, induction of angiogenesis (Xia et al., 2007; Jo et al., 2011).

Table 2. Possible mechanisms behind the induction of mutagenesis and carcinogenesis.

Mechanism	References
DNA strand breakage	Massie et al., 1972; Brawn and Fridovich, 1981; Hazlewood and Davies, 1996
Covalent binding to DNA in order to produce DNA adducts	Hazlewood and Davies, 1996
The generation of DNA-protein cross-links	Lesko et al., 1982
The generation of DNA interstrand cross-links	Lesko et al., 1982
The activation of oncogenes	Plaine, 1955
The inactivation of tumour suppressor genes	Plaine, 1955
DNA base modification and/or destruction	Massie et al., 1972

There is evidence that biomolecule damage caused by free radicals is involved not only in tumour initiation but also in later stages of the disease (Dreher and Junod, 1996). For example, in 1984, Zimmerman and Cerutti published their investigation showing that ROS can act as tumour promoters.

The most known carcinogens include polycyclic aromatic hydrocarbons which induce DNA strand breakage and other DNA damage *in vitro* and *in vivo* (Lorentzen and Ts'o, 1977; Marczynski et al., 2009). Tobacco, alcohol and their metabolites are also associated with the development of certain types of cancer.

2.3. The role of oxidative stress in the development of cardiovascular diseases

Cardiovascular diseases are a major cause of death globally. There are coronary heart diseases, cerebrovascular and peripheral artery disorders, hypertension, heart failure and congenital heart problems. A vast majority of acquired cardiovascular diseases are a consequence of atherosclerosis characterized by a local thickening of artery wall. The thickening is a result of the local accumulation of cholesterol and other fatty materials accompanied by inflammation. In addition to high serum cholesterol concentrations or dyslipidemia, also smoking, obesity, physical inactivity, hypertension, diabetes and immunological factors are, amongst others, risk factors for atherosclerosis (Garelnabi, 2010).

In animal studies it was shown that oxidative stress may play an important role in vascular aging promoting inflammation in veins (Mármol et al., 2007). *In vivo* experiments on mice have also shown that oxidative stress makes certain arterial regions susceptible to atherosclerosis possibly through the increased expression of NADPH oxidase that stimulates the production of reactive oxygen species (Haidari et al., 2010). At the same time, cardiac sensitivity to those species increases with age, which may partially be due to extracellular non-protein-bound iron (Tanguy et al., 2003). Auto-oxidation of cholesterol to epoxides and other oxygenated sterols may damage the arteries and thus play an important part in the development of atherosclerosis (Imai et al., 1980).

Recently it was shown that the deficiency of some trace metals required for the construction of antioxidative enzymes may deteriorate the cardiac function in patients with heart failure (Hiraoka et al., 2011). Particularly selenium deficiency is an important factor causing the impairment of antioxidant defence mechanisms in coronary vessels.

2.4. The role of oxidative stress in the development of neurodegenerative diseases

Neurodegenerative diseases are conditions resulting from degeneration and/or death of nerve cells. These conditions are incurable and progressive. The most common neurodegenerative disorders are Parkinson's disease and Alzheimer's disease. Typical symptoms of the latter include gradual memory loss and other cognitive impairments. In later stages of the disease various physical problems can develop. In Parkinson's disease the death of dopamine-generating cells result first in motor symptoms such as rigidity and shaking, but later also cognitive and behavioural impairments are common.

Many different mechanisms related to oxidative stress can be involved in the development of neurodegenerative disorders. For example, in Parkinson's disease increased lipid peroxidation, as well as oxidative DNA and protein damage is observed in substantia nigra, the brain area that plays a major role in the development of Parkinson's disease (Dexter et al., 1994; Alam et al., 1997a; Alam et al., 1997b). Some of those observed effects may also be due to the medicines used, especially levodopa (Alam et al., 1997a). However, recent studies show that oxidation of biomolecules in substantia nigra increases during normal ageing, while antioxidant defence weakens, which may together make the brain more vulnerable to the disorder (Venkateshappa et al., 2012).

In Alzheimer's disease, there have been also observed signs of oxidative damage to proteins, lipids and DNA (Lyras et al., 1997; Pratico et al., 1998). In addition, the senile plaques which are typical to Alzheimer's disease may also have pro-oxidative effect due to the presence of transition metal ions such as iron, copper and zinc (Lovell et al., 1998).

Huntington's disease is another progressive neurodegenerative disorder characterized by changes in personality, cognition and motor control (Walker, 2007). Compared with healthy subjects, patients having Huntington's disease and asymptomatic disease gene carriers have higher plasma lipid peroxidation levels and lower antioxidant defence (Klepac et al., 2007). Thus oxidative stress may contribute to the onset of the symptoms but also to the progression of the disease.

2.5. Other disorders associated with oxidative stress and oxidative damage

Cardiovascular, neurodegenerative and oncological diseases are likely to be the most studied disorders associated with oxidative stress. Yet oxidation can occur in all metabolically active, living cells, and therefore oxidative stress is also associated with many other common disorders and conditions. The examples are given in Table 3. Possible mechanisms behind the development and progression of such conditions are diverse. For example, lungs, eyes and skin are naturally exposed to relatively high amounts of oxygen as well as to air pollutants which makes them vulnerable to oxidative damage. Heavy metals such as cadmium increase the generation of reactive oxygen species and thus can promote cell death (Kim et al., 2008). However, the deficiency in antioxidant defence also plays an important part. Thus the deficiency in exogenous antioxidants vitamin A, vitamin E and selenium can cause problems with ocular tissues (Hayes, 1974; Katz et al., 1982).

Sometimes it can be difficult to determine if oxidative or nitrosative stress is a cause or a consequence of a disorder. However, it is possible, at least in some cases, to assess the stage and to predict the course of the disease on the basis of oxidative stress biomarkers (Su et al., 2009).

Table 3. Examples of disorders and conditions associated with oxidative and/or nitrosative stress in addition to ageing, cancer, cardiovascular and neurodegenerative diseases.

Disorder / condition	References
Autoimmune disorders such as systemic lupus erythematosus	Turgay et al., 2007
Chronic pulmonary disorders such as asthma and COPD (decreased lung function, hypoxia)	Cho and Moon, 2010; Liu et al., 2011
Deterioration / loss of hearing	Kim et al., 2008
Diabetes and its complications	Yang et al., 2011
Eye disorders, such as xerophthalmia, keratomalacia and night blindness	Hayes, 1974; Katz et al., 1982
Generalized anxiety disorder	Khanna et al., 2012
Hepatitis	Venturini et al., 2010
Inflammatory bowel diseases	Kruidenier et al., 2003
Pancreatitis	Escobar et al., 2012
Periodontal diseases	Su et al., 2009
Rheumatoid arthritis	Desai et al., 2010

3. ANTIOXIDANTS

Living organisms are capable of producing various compounds as a part of their antioxidant defence. Naturally occurring antioxidants include a range of enzymes (for example superoxide dismutase, gluthatione peroxidase), coenzyme Q, melatonin, iron-binding proteins (for example transferrin, lactoferrin), vitamins C and E as well as carotenoids, flavonoids and other plant phenolics (Halliwell and Gutteridge, 2007). There exist also numerous synthetic antioxidants such as gluthatione donors, superoxide dismutase and catalase mimetics, derivatives of vitamins E and C, N-acetyl-cysteine, xanthine oxidase inhibitors and various lipid-soluble chain breaking and transition metal binding compounds.

Some compounds possessing antioxidant activity are in clinical use for the treatment of various conditions, although they were not primarily developed as antioxidants (Halliwell and Gutteridge, 2007). For example, sulfasalazine and its active metabolite 5-aminosalicylic acid used in the treatment of inflammatory bowel diseases exert free radical scavenging activity (Joshi et al., 2005). Reactive species formed from sulfasalazine and 5-aminosalicylic acid oxidation can be scavenged by vitamin C.

As the knowledge on the role of oxidative stress in various disorders and on the effects of antioxidants accumulates, it becomes more obvious that some antioxidants could be used (possibly after some structural modifications) as adjuncts to other treatments. In addition, antioxidant compounds are used in the preservation of food. Synthetic antioxidants are popular because of their effectiveness and relatively low price. However, there have been some concerns about their safety in long-term consumption. Several investigations have shown that certain synthetic antioxidants may increase the risk of cancer at least in animals (IARC 1986). That is why natural antioxidant sources bear an increased interest not only as potential medicinal agents, but as food preservatives, too.

3.1 Mechanisms of action of antioxidants

As mentioned previously, antioxidants are compounds that can fight oxidative and nitrosative damage to a given molecule and/or tissue in many different ways. Halliwell and Gutteridge (1990) list a number of the mechanisms of action of antioxidants. They are free radical scavenging, quenching, transition metal chelation, converting free radicals into non-radical products, decreasing localized O_2 concentrations and chain breaking through reaction with chain-propagating radicals. The action of an antioxidant can be based on hydrogen or electron donation to the radical. Another important mechanism is sequential proton loss electron transfer (SPLET) which was shown to play an important part in the antioxidant action of vitamin E (Musialik and Litwinienko, 2005).

Free radical scavenging antioxidant molecules often “sacrifice” themselves, and hence the level of antioxidant(s) decreases, when oxidation proceeds (Huang et al., 2005). For example, vitamin E, or α-tocopherol, is a chain-breaking antioxidant which becomes a radical itself when reacting with free radicals (Burton and Ingold, 1986). Ascorbic acid is able to regenerate oxidized α-tocopherol to its efficient form and thus it can prolongate its antioxidant activity.

Quenching of singlet oxygen through the energy tranfer is known to be a mechanism of action of carotenoids, in particular β-carotene (Foote and Denny, 1968). Carotenoids turn oxygen back to its ground state and become excited (Figure 2). When the excess energy is lost in the interactions with the environment, carotenoid molecule is ready to quench new singlet oxygen molecules (Krinsky, 1998).

$$^1O_2 + CAR \rightarrow {}^3O_2 + {}^3CAR$$
$$^3CAR \rightarrow CAR + heat$$

Figure 2. The mechanism of quenching singlet oxygen (Krinsky, 1998). $^1O_2$ = singlet oxygen (highly reactive), $^3O_2$ = ground state oxygen, CAR = ground state carotenoid, ^{3}CAR = carotenoid in its excited state

Chelating transition metal ions, especially iron, is an important mechanism of antioxidant action, because free metal ions can promote oxidation by catalysing free radical reactions. Antioxidants acting in this manner are usually termed preventive antioxidants. When testing the ability of samples to chelate metal ions, it is important to consider the efficiency of such mechanism as a part of antioxidant defence. The chelation is beneficial in reducing oxidative stress only if the bound metal ions do not continue to participate in redox reactions. For example, EDTA effectively binds iron, but the complex formed in this reaction can catalyse oxidative damage even more than free iron (Brawn and Fridovich, 1981; Aruoma et al., 1989). However, such chelates may prove effective in killing cancer cells.

Although it is well known that free radicals play a part in tumour initiation and promotion, recent investigation has shown that the excess addition of reactive oxygen species or molecules producing them inhibits the proliferation of tumour cells and results in cell death (Laurent et al., 2005). According to the investigators the increased production of reactive oxygen species is likely to be a possible mechanism of action of anticancer medicines such as oxaliplatin. Such observations make it quite possible that reactive complexes of transition metals and their chelators mentioned above exert antitumour activity and could be developed into chemotherapeutics for the treatment of cancer.

It is possible, that at least some antioxidants have more than one mode of action. For example, uric acid is known to be a chain-breaking free radical scavenger, but it is also capable of chelating transition metal ions thus inhibiting the initiation of oxidation chain (Ames et al., 1981; Davies et al., 1986). In the absence of singlet oxygen, β-carotene inhibits radical-initiated reactions by the mechanism different from that of preventive and conventional chain-breaking antioxidants (Burton and Ingold, 1984). On the other hand, vitamin E is known to employ quenching in addition to its scavenging activity (Fahrenholtz et al., 1974)

3.2. The action of antioxidants *in vivo*

The function of antioxidants is assumed to be protective, and their consumption from food and food supplements is thought to have beneficial effect on human health. For example, the phenolic compounds of fruits and berries have been found to be effective in the inhibition of human cancer cell proliferation and inducing apoptosis in tumour cell lines *in vitro* (Olsson et al., 2004; Seeram et al., 2006). In addition, there exist epidemiological data supporting the fact that the intake of antioxidant-rich foods may protect against cancer (Riboli and Norat, 2003). Although vitamin C alone may not be effective in inhibiting the growth of tumour cells, plant foods containing high vitamin C contents are able to fight cancer at least *in vitro* thus suggesting that there must be synergistic action with other components (Olsson et al., 2004).

There is also evidence that antioxidants obtained from food rich in vitamins C and E, β-carotene and flavonoids may exert a protective effect against ischemic stroke (Vokó et al., 2003). The Rotterdam Study by Vokó and colleagues (2003) suggests that especially smokers could benefit from high dietary intake of antioxidants, in particular vitamins C and E. However, according to another study on the effects of food antioxidants on the risk of the ischemic stroke, vitamin E is not effective (Hak et al., 2004). Carotenoids, on the contrary, are associated with lower stroke risk. Although there is no agreement on what antioxidant components of daily diet are the most important for cardiovascular health, regular fruit and vegetable intake can be recommended as a part of health promoting way of life.

However, the way antioxidants act in the body is complicated. For example, known antioxidants vitamin C and N-acetyl-cysteine do not heal acute muscle injury, but act as pro-oxidants promoting oxidative stress and tissue damage (Childs et al., 2001). It was already known a couple of decades ago that ascorbate can produce extensive lipid peroxidation in the presence of ferric chloride (Davies et al., 1986). This reaction can be only partly prevented by endogenous antioxidants such as uric acid. As injury can release ferric ions from damaged tissue, it could serve a possible explanation for the deleterious action of vitamin C in acute muscle injury.

Vitamin E is also known as antioxidant, but smokers are not recommended to use high-dose vitamin E supplementation, because in certain cases it may promote lipid oxidation and thus induce oxidative stress (Weinberg et al., 2001). Another example is uric acid which can be produced from dietary purines and is suggested to scavenge free radicals in human blood (Ames et al., 1981). Despite its beneficial effects, the excess of urate causes painful joint inflammation known as gout.

Noteworthy, antioxidants cooperate *in vivo* to protect the organism against radical-induced damage in the best possible way. Antioxidant defence is a combination of water-soluble (for example, ascorbate) and lipid-soluble (for example, tocopherols) antioxidants which act at different sites in the body. In most cases antioxidants are oxidized during their reaction with reactive species, but due to the cooperation with

other antioxidants they can be regenerated back to their active form as for example in case of α-tocopherol (Burton and Ingold, 1986).

3.3. Antioxidants in foods

Food products naturally contain varying amounts of antioxidants. Plant-derived foods are often thought to be the only sources of antioxidants, but in fact animal-derived foods also contain antioxidative compounds, though generally in much lower concentrations (Carlsen et al., 2010). Whatever the origin, products usually lose at least some of their antioxidant content during processing and long-term storage (Szeto et al., 2002). Using antioxidant-derived preservatives can help to prolong the shelf life of many products such as various commercial juices, sauces and fat-containing foods.

Synthetic antioxidants such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) have been used in food industry as efficient and inexpensive preservatives to prevent oxidative rancidity of fat-containing foods for decades (IARC 1986). WHO's International Agency for Research on Cancer has evaluated the carcinogenicity of BHA and BHT, and although human data were not available, data obtained from animal experiments have suggested possible role of the intake of synthetic antioxidants in cancer.

In the Netherlands Cohort Study no clear evidence of carcinogenicity in human stomach was found when daily intake of BHA and BHT was evaluated on the basis of the frequency of consumption of certain foods (Botterweck et al., 2000). The follow-up period was 6.3 years, which makes it possible that long-term consumption of synthetic antioxidants can still cause negative effects in the body. Therefore there is an increasing need for the discovery of new natural preservatives. Promising results have been achieved from the investigations on the antioxidant and antimicrobial properties of some herbs and spices (Szabo et al., 2010).

4. ANTIOXIDANTS IN PLANT FOODS

Vegetables, fruits and berries are commonly considered as an important part of healthy human diet and are even recommended by authorities for extensive daily use. Plant foods are known to be rich in vitamins and other beneficial compounds such as phenolic substances. Epidemiological studies have shown that the consumption of plant foods can have a profound protective effect against many diseases such as cardiovascular diseases and cancer (Bazzano et al., 2002; Riboli and Norat, 2003). Thus plant foods, especially those possessing antioxidative properties, become more and more important in prevention and treatment of various diseases. Vegetables, fruits and berries as an essential part of daily diet may effectively protect the organism against oxidative and nitrosative stress, hence preventing the damage of cells and biomolecules. Nevertheless, the antioxidant composition and contents may vary significantly making one plant group or even species more efficient as a protector against oxidative damage than another.

For many plant foods, especially fruits and berries, ascorbic acid is usually thought to be a major source of antioxidant power. However, the investigation on common fruits, berries and vegetables together with less common Chinese vegetables shows, that high total antioxidant power may be due to components other than vitamin C, which is the case for example with plum (Szeto et al., 2002).

In addition to vegetables, fruits, berries and herbs, there are also other antioxidant-rich foods of plant origin. Cereals, nuts (particularly walnut) and seeds contain a variable amount of antioxidants (Halvorsen et al., 2002). Those, however, will not be discussed further in this thesis.

4.1. Antioxidants in vegetables

In earlier studies, for example, broccoli and cauliflower have shown good *in vitro* antioxidant activity when compared with some pure substances such as BHT (Gülcin et al., 2004; Köksal and Gülcin, 2008). In a Chinese investigation on the antioxidant

capacity of a range of plant foods, Chinese vegetables have proven to possess better total antioxidant power than many vegetables commonly used in Europe (Szeto et al., 2002). According to the authors, loss of antioxidants could have occurred during the transport of foreign vegetables from elsewhere to China as well as during storage. Comparing vegetables commonly used in Europe, onions, turnip and cabbage seem to be the most beneficial sources of antioxidants followed by broccoli, cauliflower, garlic and tomato. The lowest antioxidant capacity is observed in lettuce, potato, celery and carrot.

In a Norwegian investigation peppers, in particular chili pepper, kale, red cabbage, parsley, artichoke leaves, brussel sprouts and spinach were the best sources of antioxidants among vegetables (Halvorsen et al., 2002). Onion, turnip and cabbage appeared to have quite low total antioxidant concentrations. On the contrary, the results for potato (except for the blue species) and carrot were consistent with the Chinese investigation mentioned above (Halvorsen et al., 2002, Szeto et al., 2002). Perhaps, the differences in the obtained results are, at least partially, due to different methods in sample preparation and analysing. The effects of transportation and storage on the antioxidant concentrations of tested plants cannot be excluded either.

Considering the average consumption of various vegetables, the best sources of antioxidants compared to their common serving size are likely to be kale, beets, red pepper, brussel sprouts, broccoli, spinach, potatoes and corn (Cao et al., 1996). However, as the serving size of various vegetables as well as the frequency of their consumption varies individually, the significance of a single vegetable as a source of antioxidant activity may differ from that of the average.

In daily diet it should also be considered that antioxidant activity of vegetables may vary after thermal treatment compared with fresh vegetables. The change is not always negative, but in fact, antioxidant activity of certain vegetables may increase after boiling (Gazzani et al., 1998). On the contrary, the processing of fresh vegetables (as well as other dietary plants) such as chopping or shredding may result in loss of antioxidants, inter alia ascorbic acid (Szeto et al., 2002). Long-term storage may also play a part in

antioxidant loss.

4.2. Antioxidants in fruits and berries

In addition to vegetables, fruits and berries tend to be the most studied antioxidant-containing foods. They contain a number of phenolic compounds with antioxidant activities, including vitamin C, anthocyanins, carotenoids, flavonols and ellagitannins (Olsson et al., 2004). The composition and the content of antioxidant-related compounds differ in various species. As it has been shown with raspberry, blackberry and blueberry, total phenol, flavonoid and anthocyanin content as well as antioxidant activity can vary significantly depending on the cultivar in question (Sariburun et al., 2010; Rodrigues et al., 2011).

High antioxidant concentrations are observed in berries, particularly in dog rose, but also in crowberry, bilberry, blackcurrant, wild strawberry, sour cherry, wild and cultivated blackberry as well as in cranberry (Halvorsen et al., 2002). A range of cultivated berries, including strawberry, are significantly lower in their antioxidant content. Most of the fruits also seem to contain much less antioxidants than berries. Among fruits, pomegranate has very high total antioxidant concentration. Weaker than pomegranate but still good sources of antioxidants are grape, orange, plum, pineapple, lemon, date, kiwi fruit, clementine and grapefruit.

The results from the investigation by Szeto and colleagues (2002) are partially consistent with those obtained by Halvorsen and colleagues (2002). Here, strawberry, lemon, plum, orange, kiwi fruit and grapefruit have greater antioxidant capacity than persimmon, apple, mandarin, mango, grapes, banana, pear and pineapple (Szeto et al., 2002). Earlier, similar order was obtained with the use of different assay method: strawberry, plum and orange were the most active in scavenging ROS, while banana, apple, pear and melon had the lowest antioxidant activity (Wang et al., 1996).

Fruits and berries exerting the greatest total antioxidant power in the investigation by Szeto and colleagues (2002) have also relatively high vitamin C content, with exception

of plum. Noteworthy, many citrus fruits are proved to be excellent sources of antioxidants by two independent investigations performed in Norway and in China (Halvorsen et al., 2002; Szeto et al., 2002).

4.3. Antioxidants in herbs and spices

As an important source of antioxidants, herbs and spices are considered to have a great potential as food preservatives (Hinneburg et al., 2006; Szabo et al., 2010). For example, the extract obtained from a mixture of culinary herbs (namely milfoil, rosemary, marjoram, thyme, lovage, oregano and basil) has proven to be potential preservative which may replace notorious synthetic antioxidants such as BHT at least in some food products (Szabo et al., 2010). Unfortunately, strong aroma of natural herb-derived antioxidants may restrict their use at some extent. In addition, the duration of antioxidant activity may be limited to less than two years (Szabo et al., 2010).

In addition, herbs are widely used in traditional medicine particularly widespread for example in China. The determination of the total phenolic content and antioxidant-related activity of such medicinal herbs revealed that most of them are much stronger potential antioxidants than dietary fruits and vegetables (Cai et al., 2004). Dietary herbs seem to be remarkable sources of antioxidants as well (Dragland et al., 2003). However, there can be significant differences among the antioxidant concentrations in various species.

In the research of Dragland and colleagues (2003) dried greenhouse herbs containing the highest levels of antioxidants became arranged in the next order (from higher to lower concentrations): oregano, sage, peppermint, thyme and lemon balm. When commercial dried spices were tested, the order was different with rosemary and thyme having higher antioxidant content than oregano and many other spices, and total antioxidant concentrations were mostly lower than those of dried culinary herbs perhaps due to different varieties used as well as different drying and storage conditions. In addition, researches can give distinct results and order for the same herbs and spices due to the methods and conditions used.

The antioxidant concentration may vary not only in different plant species, but also in different varieties of the same plant (Daood et al., 1996; Dragland et al., 2003). There may be significant seasonal variations as well, thus it can be difficult to compare the antioxidant activities of herbs harvested at different times of the year or even in different years (Dragland et al., 2003). Plant habitat can also influence the concentrations of active compounds.

Studies have shown that the antioxidant capacity of herbs do not decrease with drying, especially when air-drying methods is used (Hossain et al., 2010). On the contrary, fresh herbs seem to lose phenolic compounds and their antioxidant-related activities due to enzymatic degradation and atmospheric oxygen promoted oxidation. That is why herbs dried with an appropriate technique are suitable both for providing the organism with antioxidants and for the preservation of certain foods. The situation may be different with spices such as paprika, which is obtained from drying and grounding red pepper fruits. Paprika loses its antioxidant content, primarily vitamins C and E, during drying process (Daood et al., 1996). In addition, antioxidative properties of ground paprika deteriorate in a few months of storage. Thus antioxidant intake from spices may sometimes be quite uncertain.

5. METHODS IN STUDYING ANTIOXIDANT-RELATED ACTIVITIES

Antioxidant-related substances have been studied in many different ways *in vitro* and *in vivo*. Their activity has been evaluated in test tubes, in cell cultures, in animals and in humans, in healthy individuals as well as in patients or disease models. In this thesis I am going to briefly focus only on the *in vitro* methods.

In vitro antioxidant activity can be determined by measuring the substrates, the oxidants, the initiators (including transition metal ions), the intermediates or final products of oxidation reaction (Antolovich et al., 2002). The most usually used methods are based on the assessment of the capability of an antioxidant to inhibit the production of oxidative intermediates and final products and thus to prevent oxidative damage.

According to Huang and colleagues (2005) major antioxidant capacity assays can be roughly divided into hydrogen atom transfer reaction based and single electron transfer reaction based assays (Table 4). The exact division of the methods into two categories can be difficult, because there exist also mechanisms such as proton-coupled electron transfer. However, in both the hydrogen and the electron transfer based mechanisms it is the free radical scavenging capacity that is assessed, not the preventive one.

Table 4. Methods used in studying antioxidant-related activities can be roughly divided into two categories depending on the mechanisms involved.

Hydrogen atom transfer reaction based	Single electron transfer reaction based
Conjugated diene formation	Total phenols assay
Oxygen radical absorbance capacity (ORAC) assay	Trolox equivalent antioxidant capacity (TEAC) assay
Total radical-trapping antioxidant parameter (TRAP) assay	Ferric ion reducing antioxidant power (FRAP) assay
Crocin bleaching assay	N,N-dimethyl-*p*-phenylenediamine (DMPD) assay
(1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging capacity assay)	1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging capacity assay
	Cu(II) reduction capacity assay
	Reducing power activity assay

5.1. Hydrogen atom transfer reaction-based assays

The capacity of antioxidant to inhibit the induced lipid auto-oxidation can be evaluated by measuring conjugated diene formation (Antolovich et al., 2002; Huang et al., 2005). As an initiator of oxidation either an azo compound or a transition metal (for example, copper) can be used. An antioxidant is added at certain point of the reaction, and the reaction slows for the time corresponding to the concentration and capacity of the antioxidant. Reaction kinetics can be monitored using UV spectrometer or alternatively (and less desirably) gas chromatography (Huang et al., 2005).

There exist several colorimetric and fluorometric assays measuring the inhibition or the delay of substrate oxidation using a competitive antioxidant (Huang et al., 2005). In addition to an antioxidant and a molecular UV or fluorescent probe, such assays use an azo radical to initiate the reaction. Examples include ORAC assay and TRAP assay.

The ORAC assay is a suitable method for measuring the antioxidant capacity of hydrophilic and lipophilic compounds against peroxyl radicals (Huang et al., 2005). In this assay samples are incubated with a fluorescent probe at neutral pH after which radical initiator is added to start the reaction. The progression of the reaction is monitored spectrophotometrically as fluorescence attenuation which is inhibited by an antioxidant in accordance with its chain-breaking capacity.

The TRAP assay developed by Wayner and colleagues (1985) is one the most used methods for the evaluation of antioxidant status of biological fluids. The principle is similar to that of the ORAC assay. An azo compound is used as an initiator of peroxidation, and the so called induction period (the time before the antioxidant becomes ineffective in inhibiting the initiation of peroxidation) is measured (Wayner et al., 1985).

Less used methods include crocin bleaching assay in which the discolouration of the reaction mixture is monitored by an UV-vis spectrometer (Huang et al., 2005). Crocin is a mixture of natural carotenoid pigments, and its lot-to-lot variability limits the use of the method.

5.2. Electron transfer reaction-based assays

Assays based on the electron transfer include total phenols assay performed with the use of Folin-Ciocalteu reagent. This is one of the most often used assays to indicate hydrophilic antioxidant potential. Folin-Ciocalteu reagent measures a reducing capacity of a sample (Huang et al., 2005). Phenolic compounds reduce the reagent under basic conditions, which obtains the colour change and can be detected spectrophotometrically.

The DPPH free radical scavenging assay is based on the use of a violet coloured nitrogen-centred free radical 1,1-diphenyl-2-picrylhydrazyl (also known as α,α-diphenyl-β-picrylhydrazyl), DPPH (Huang et al., 2005). The assay was believed to involve the donation of hydrogen to DPPH and a consequent reduction of free radical which would change its violet colour to yellow. Recently, however, it was shown, that in methanol and other strong hydrogen bond-accepting solvents the major mechanism is likely to be the electron transfer (Foti et al., 2004). The assay can be performed either over time or in fixed time manner. The latter approach is used to simply compare the free radical scavenging activities of samples, while the previous one gives information about the reaction kinetics.

The TEAC assay is a simple and widely used method for studying the antioxidant capacity of both hydrophilic and lipophilic samples (Huang et al., 2005). The oxidant solution is mixed with a sample, and the absorbance is measured at several time points. The discolouration of an oxidant in the presence of antioxidant causes the change in absorbance which is compared to the standard to assess the antioxidant capacity. The principle of FRAP assay is quite similar to that of TEAC assay, but different reagents and conditions are used. In FRAP assay ferric ion may interfere with the accurate measurement of antioxidant capacity, which may limit its application in studying food extracts.

The reducing power activity assay, also known as iron reduction assay, is based on the ability of phenolic compounds to reduce potassium ferricyanide to ferrous state, which can be detected spectrophotometrically (Dorman et al., 2003). Reducing power is determined from the intensity of obtained Prussian blue colour. The bigger the concentration and the reducing power of the sample is, the higher is the absorbance measured.

5.3. Other methods

There exist also assays evaluating the capacity of a sample to scavenge specific ROS such as singlet oxygen or hydroxyl radical (Huang et al., 2005). So far many of those

assays have disadvantages of being unable to provide meaningful data, because the observed activities may result from mechanisms other than the actual scavenging of an oxidant. Some of the methods can also be relatively complicated and time-consuming, thus requiring modifications before they can be applied more widely.

The most important method in detecting lipid oxidation is thiobarbituric acid reactive substances (TBARS) assay measuring the malondialdehyde (MDA) formation (Antolovich et al., 2002). A transition metal ion or a free radical source is used to oxidize the substrate (for example, LDL or tissue sample). The resulting MDA reacts with thiobarbituric acid to form a coloured complex, which is measured spectrophotometrically. In the presence of antioxidant the complex formation and hence the absorbance are reduced.

Iron (II) chelation assay can be used to assess the preventive antioxidant capacity of a sample. It is based on the reaction of ferrozine with ferrous ions (Stookey, 1970). This reaction leads to the formation of coloured complex and is disrupted by the chelation of ferrous ions. The higher is the concentration of chelating sample, the less ferrous ions is left to react with ferrozine, which can be detected spectrophotometrically as the reduction of absorbance.

5.4. Advantages and possible drawbacks

The most widely used methods in studying antioxidant-related activities are relatively cheap and relatively easy to perform, and required equipment is commonly found in general purpose laboratories. There can be some problems with solubility, which can be seen, for example, as precipitation (for example, in reducing power activity assay) or opacity (for example, in iron chelation), especially in strong samples. Spectral interference is another possible issue to take into account when using coloured samples, which may absorb UV irradiation at the same wavelengths as reagents used to detect activity. The possibility of such interference can be checked with scanning the absorption of UV light by a given sample at different wavelengths.

5.5. Results and correlations

Results obtained in different investigations sometimes have poor correlation, especially when comparing *in vitro* results with *in vivo* ones. Living organism is more complex than *in vitro* experimental models, because it contains various co-operating systems that cannot be recreated in a tube. There may exist various compensatory and recovery mechanisms *in vivo* allowing to overcome the deficiency of antioxidative substances and the damage caused by such deficiency and excessive activity of oxidants. The problem with *in vivo* results may lie in differences between species and individuals concerning metabolic and genetic variations. There may be synergistic action between various compounds of the same plant or of two different plants in the body, as well as interactions between exogenous and endogenous compounds. That is why testing a single compound *in vitro (*or *in vivo)* cannot provide complete information on the activity of a given plant. It is also important to take natural result variation into account.

II. EXPERIMENTAL

6. MATERIALS AND METHODS

6.1. Objective

The objective of performed experiments was to evaluate the antioxidant-related properties of some commonly used culinary plants. The evaluation was based on four distinct *in vitro* procedures (See 6.5. Methods) to obtain versatile information on the potential antioxidant efficacy of the samples. The aim of this research was not to determine which plants are more beneficial in daily diet in preventing or treatment of various conditions, but to provide a clue which commonly used vegetables, berries, herbs and spices could be valuable for further closer research.

6.2. Materials

The samples used in this research are presented in Table 5. Vegetables and berries were purchased from local supermarket. Herbs and spices were obtained from Paulig Oy (Finland). Vegetables (broccoli, brussel sprouts, cauliflower and peas) and berries (bilberry and raspberry) were reduced in size, where appropriate, and frozen. Air-dried Egyptian basil, oregano, rosemary and thyme were used as supplied. Paprika was obtained dried and ground.

Food samples were chosen for this research on the basis of their common use and the absence of sufficient published research, especially on the phenolic content. Bilberry and paprika have been researched at some extent but not very extensively. Basil, oregano, rosemary and thyme are well known herbs which have been studied, but not with the extraction method and solvents used in this study.

To ensure that the assays would work as expected and to compare the activities of the samples to known compounds, a number of pure substances (gallic acid, vitamin C, BHT, quercetin and Pycnogenol) were tested.

Table 5. Vegetables, berries, herbs and spices tested.

Common name	Latin name	Family
Bilberry	*Vaccinium myrtillus* L.	Ericaceae
Broccoli	*Brassica oleracea* L. var. *italica* Plenck	Brassicaceae
Brussel sprouts	*Brassica oleracea* L. var. *gemmifera* (DC) Zenker	Brassicaceae
Cauliflower	*Brassica oleracea* L. var. *botrytis*	Brassicaceae
Egyptian basil	*Ocimum basilicum* L.	Lamiaceae
Oregano	*Origanum vulgare* L.	Lamiaceae
Paprika	*Capsicum annuum* L.	Solanaceae
Peas	*Pisum sativum* L.	Fabaceae
Raspberry	*Rubus idaeus* L.	Rosaceae
Rosemary	*Rosmarinus officinalis* L.	Lamiaceae
Thyme	*Thymus vulgaris* L.	Lamiaceae

Purified water used as a solvent for the samples and reagents was prepared using a Millipore Milli-RO 12 plus system. Other solvents were obtained either from Rathburn Chemicals Ltd (Walkerburn, Scotland) or from Merck KGaA (Darmstadt, Germany). Reagents were obtained from Sigma-Aldrich (St. Louis, MO, USA), except for Pycnogenol which was obtained from Biolandes Arômes (Boulogne, France) and quercetin which was obtained from Extrasynthèse (Genay, France). All reagents and solvents were of analytical or HPLC grade and were used without further purification.

6.3. Equipment

Apparatus used in the preparation of samples and certain reagents are presented in Table 6. Glassware included test tubes, measuring cylinders, separatory funnels, round-bottom flasks, beakers, Erlenmeyer flasks and volumetric flasks of various sizes. At different stages of the work, samples were stored either in glass bottles with plastic tops or in appropriate laboratory glassware covered with marbles and/or Parafilm M (Brand GmbH + Co.KG, Wertheim, Germany). Büchner funnels and filter paper Whatman №4 and №2 (Whatman International Ltd., Maidstone, England) were used for the filtration of the extracts, as required. In addition, Eppendorf pipettes of various volumes, Pasteur pipettes, spoons and spatulas were used. For the absorbance measurement, samples were transferred into plastic disposable microcuvettes (1.5 ml) (Brand GmbH + Co.KG,

Wertheim, Germany).

Table 6. Apparatus used in this work.

Apparatus	Manufacturer
Unicam UV500	Unicam, Cambridge, UK
Vortex-Genie® 2 G-560E	Scientific Industries, NY, USA
VWR Ultrasonic Bath	VWR International bvba/sprl, Leuven, Belgium
Ohaus® AS120 and Ohaus® C305-S laboratory balances	Ohaus Corporation, Florham Park, USA
Schott Geräte pH meter CG820	Schott-Geräte GmbH, Hofheim a. Ts, Germany
Grant OLS200 combined orbital / linear shaking bath	Grant Instruments, Cambridge, UK
Heidolph VV2000 rotary evaporator with Heidolph WB2000 water bath	Heidolph Instruments GmbH & Co.KG, Schwabach, Germany
Heto LyoPro 3000 freeze dryer	Heto Holten A/S, Allerød, Denmark
IKA® A11 basic analytical mill	IKA-Werke GmbH & Co.KG, Staufen, Germany

6.4. Sample preparation

Extracts were prepared with the method modified from Debnath et al. (2011) (Figure 3). Broccoli, brussel sprouts, cauliflower, peas and raspberry were chopped. All vegetables and berries were freeze-dried and powdered. After weighing the powders, appropriate amount of 80 % (v/v) methanol was added and samples were sonicated for 20 minutes. For herbs and spices as well as for peas and raspberry the methanol extraction ratio was 1:10 (w/v), while for other samples it was 1:20 (w/v). After sonication, samples were filtrated using Büchner funnel with Whatman №4 for vegetables and berries and Whatman №2 for herbs and spices. The marc was re-extracted with 80 % (v/v) methanol followed by filtration, and the resulting liquid phases were combined. Brussels sprouts, cauliflower and peas extracts were additionally filtrated with Whatman №2. Methanol was removed from the samples in vacuum at maximum 40 °C using rotary evaporator and the volume was adjusted to *ca*. 200 ml with distilled water. Liquid-liquid extraction with hexane was performed thrice for herbs, broccoli and

brussel sprouts samples to remove chlorophyll and other lipophilic compounds. The resulting aqueous phase was reduced in vacuum rotary evaporator at 40 °C, frozen and lyophilised. Samples were stored in dark place at 4 °C until required and then they were dissolved in distilled water prior the use and diluted to appropriate concentrations for testing.

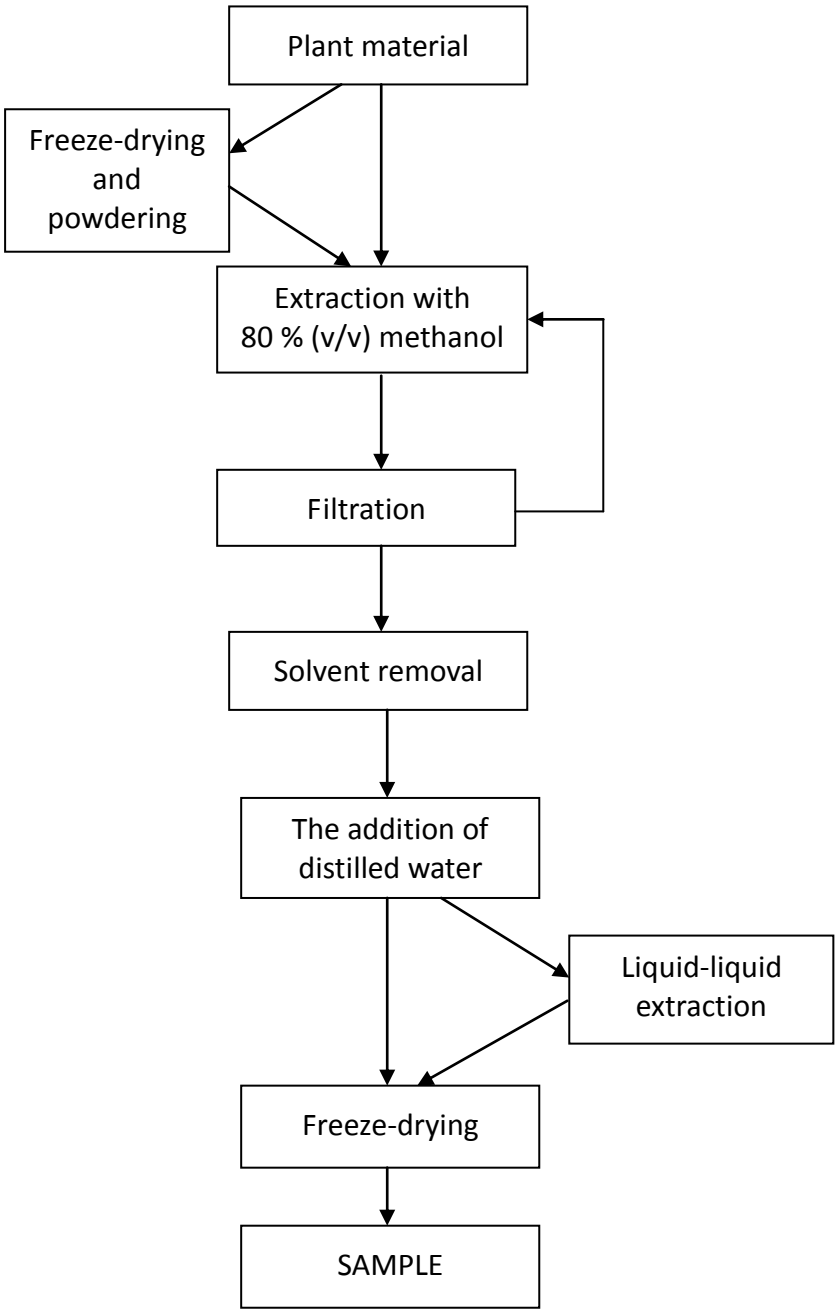

Figure 3. The scheme illustrating sample preparation procedure.

6.5. Methods

6.5.1. The determination of total phenols

The determination of total phenolic compounds was performed using modified method described by Zhang and colleagues (2011). Fifty microliters of sample or reagent blank (water) was pipetted into test tube and then the same amount of Folin-Ciocalteu reagent was added. Samples were vortexed for ten seconds and left at room temperature for two minutes, after which 500 µl of 5 % (w/v) sodium carbonate solution was added to stop the reaction. Four hundred microliters of distilled water was added to make up the volume to 1 ml. Samples were rapidly vortexed and left at room temperature for 30 minutes. Absorbance was measured at 760 nm.

First, gallic acid standards in concentrations ranging from 5 to 300 µl/ml were tested to obtain the calibration curve, after which samples were tested at concentrations of 0.5 and 1 mg/ml. All samples were analysed in five replicates. Gallic acid equivalents were calculated for each sample using the equation of calibration curve and the mean absorbance of 1 mg/ml sample when possible.

6.5.2. The DPPH free radical scavenging activity assay

DPPH free radical scavenging activity assay was performed in fixed time manner using the method described by Gyamfi and colleagues (1999). First, 50 µl of sample or control (water) and 450 µl of 50 mmol/l Tris-HCl buffer (pH 7.4) was pipetted into test tube and swirled. Then 1.0 ml of 0.1 mmol/l DPPH-methanol solution was added, the mixture was swirled and kept in a dark place for 30 minutes. After incubation period, absorbance was measured at 517 nm with the mixture of water, buffer and methanol as blank solution, and obtained values were converted into percentage inhibition using the equation (1):

(1) $$\text{Inhibition \%} = [(Abs_{ctrl} - Abs_{sample})/Abs_{ctrl}] \times 100\ \%,$$

where Abs_{ctrl} is the absorbance of a control and Abs_{sample} is the absorbance of a sample.

6.5.3. Reducing power activity assay

Reducing power activity assay was performed based on the method used by Dorman and colleagues (2003) with slight modifications. One hundred microliters of sample or reagent blank (water) was pipetted into test tube. Two hundred fifty microliters of 0.2 mol/l sodium phosphate buffer and the same amount of 1.0 % (w/v) aqueous potassium ferricyanide solution were added and swirled. Test tubes were covered with marbles to prevent evaporation and placed into 50 °C water bath for 30 minutes. After the incubation period, 0.25 ml of 10 % (w/v) trichloroacetic acid solution was added, and samples were vortexed, then 0.625 ml of sample was transferred into clean test tube. Finally, 0.625 ml of distilled water and 0.25 ml of 0.10 % (w/v) ferric chloride solution were added, after which sample was vortexed and absorbance was measured immediately at 700 nm. On the basis of obtained absorbance values, ascorbic acid equivalents were calculated.

6.5.4. Iron (II) chelation

Iron (II) chelation assay was performed according to the method used by Jimenez-Alvarez and collegues (2008). Briefly, 0.675 ml of sample and 0.075 ml of iron (II) solution were pipetted into test tube, vortexed and allowed to react for 20 minutes. Then 0.75 ml of ferrozine solution was added, samples were vortexed again and allowed to stand for another 5 minutes. Absorbance was measured at 562 nm. EDTA standards at concentrations ranging from 0 to 100 µM were used to obtain calibration curve, and the absorbance was measured against blank solution containing buffer, water and ferrozine solution. Samples were tested at concentrations of 0.5 and 1 mg/ml, and as blank solution the mixture of 0.75 ml of water and 0.75 ml of ferrozine solution was used. All samples were tested in five replicates. EDTA equivalence values were calculated for test samples using the calibration curve and the mean absorbances for 1 mg/ml concentration when possible.

6.5.5. Statistical methods

All samples were tested in five replicates using four methods described above. Mean values were used for further calculations and the interpretation of the data. In addition, standard deviation, standard error of the mean and 95 % confidence interval were calculated for each sample to assess the accuracy and reliability of the results. The calculations were performed using Microsoft Excel 2010. For values used in the calculations and for statistical data see Appendix 1, 2, 3 and 4.

7. RESULTS

7.1. The determination of total phenols

The standard curve which was obtained from gallic acid standards is presented in Figure 4. Gallic acid equivalents for each sample were calculated using the equation of this gallic acid standard curve. The results are presented in Table 7. The lower is the value, the less total phenols there is in the sample.

The smallest total phenolic content is observed in vegetable samples, especially in peas. On the contrary, herbs, in particularly thyme and oregano, have the highest content of total phenols in them. The total phenols of rosemary could not be determined, but on the basis of the research conducted by Wojdylo and colleagues (2007) it can be assumed to have even higher level of phenols than oregano and thyme. The results may vary to some extent because of different sample preparation procedure and determination method used.

Although oregano and thyme had the highest level of phenolics in this research, earlier studies reveal that their total phenolic content is actually quite low compared to some other dietary and medicinal herbs such as *Echinacea purpurea, Valeriana officinalis* and *Melissa officinalis* (Wojdylo et al., 2007).

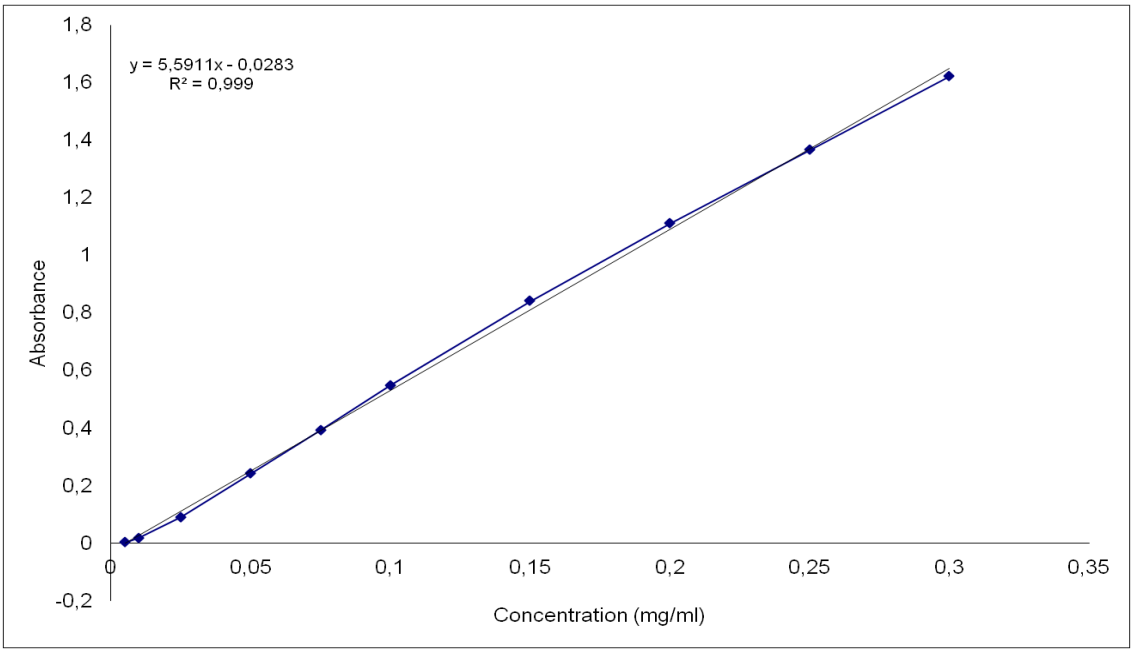

Figure 4. Gallic acid standard curve used for the calculation of gallic acid equivalents for tested samples.

Table 7. Gallic acid equivalents for tested samples.

Sample	mg GA / g sample
Bilberry	0.091 ± 0.007
Broccoli	0.018 ± 0.005
Brussel sprouts	0.020 ± 0.005
Cauliflower	0.010 ± 0.005
Egyptian basil	0.054 ± 0.006
Oregano	0.178 ± 0.007
Paprika	0.065 ± 0.005
Peas	0.007 ± 0.005
Raspberry	0.026 ± 0.005
Rosemary	nd
Thyme	0.340 ± 0.010

Values presented as mean value ± standard deviation (n = 5). nd = Not determined.

7.2. The DPPH free radical scavenging activity assay

Percentage inhibition for every tested concentration of every sample was calculated and is presented in Appendix 2. IC_{50} values are compared in Figure 5. The smaller IC_{50} value, the more active is sample in scavenging the DPPH free radical. As can be seen from Figure 5, vegetables are less active as free radical scavengers than other tested samples. Peas sample is the less active one. Broccoli, brussel sprouts and cauliflower possess about the same activity. The best free radical scavenging activity is observed in rosemary, thyme, oregano and bilberry.

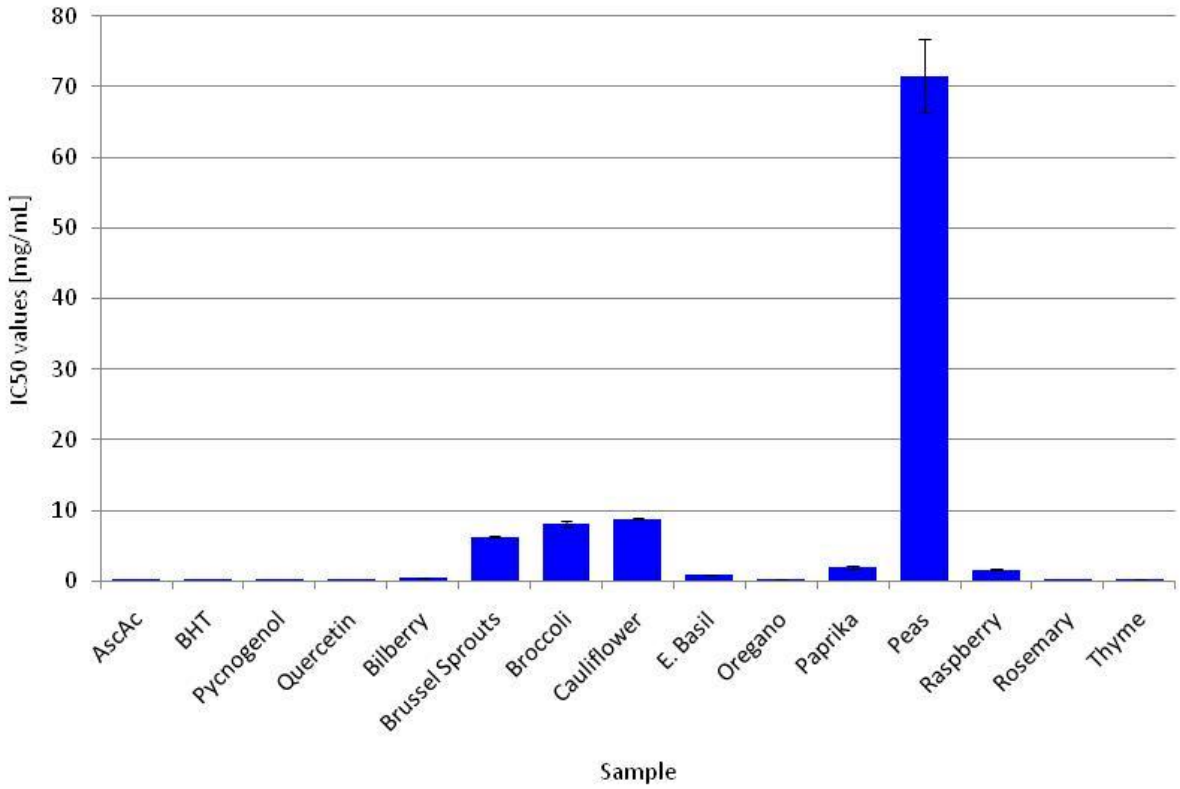

Figure 5. The DPPH free radical scavenging activity of tested samples compared with pure antioxidant substances. AscAc = ascorbic acid. BHT = butylated hydroxytoluene

7.3. Reducing power activity assay

Linear curves are obtained in this assay (Figure 6). The lower is the curve, the less reducing potential the sample has. In concordance with the previous assays, the peas

sample is the less active one. Other vegetables do not possess great reducing potential either. Rosemary, thyme, oregano and bilberry have shown the best reducing activity. However, when compared with pure substances, tested samples seem to be weak iron reducers (Figure 6). At least, none of the samples is as good in iron reduction as gallic acid, ascorbic acid, quercetin, BHT and Pycnogenol.

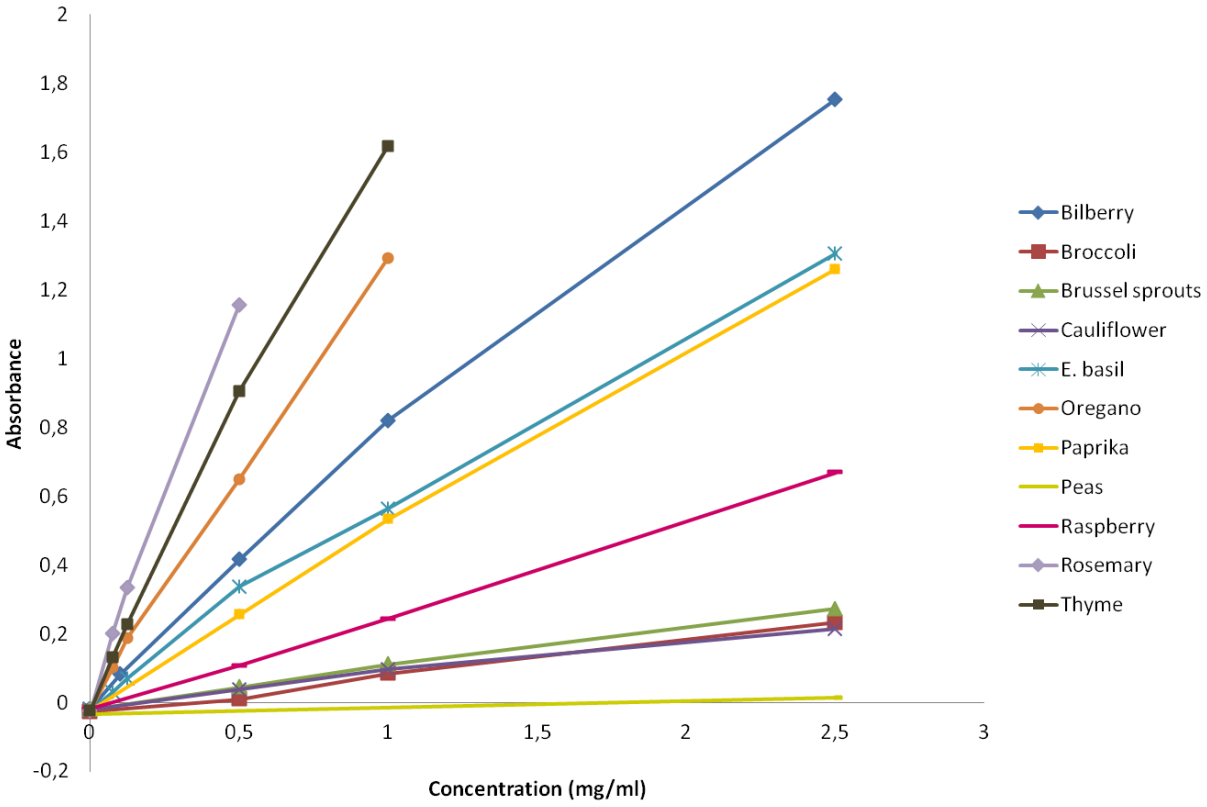

Figure 6. The results obtained from the reducing power activity assay. The lower the curve, the less active is the sample.

7.4. Iron (II) chelation

EDTA standard curve was obtained from EDTA standards in micromole concentrations and was used to calculate EDTA equivalents for tested samples (Figure 8). The bigger is the value, the better the ability of a given sample to chelate ferrous ions is. In Figure 9 the EDTA equivalents of tested samples are compared with ones of pure antioxidant substances.

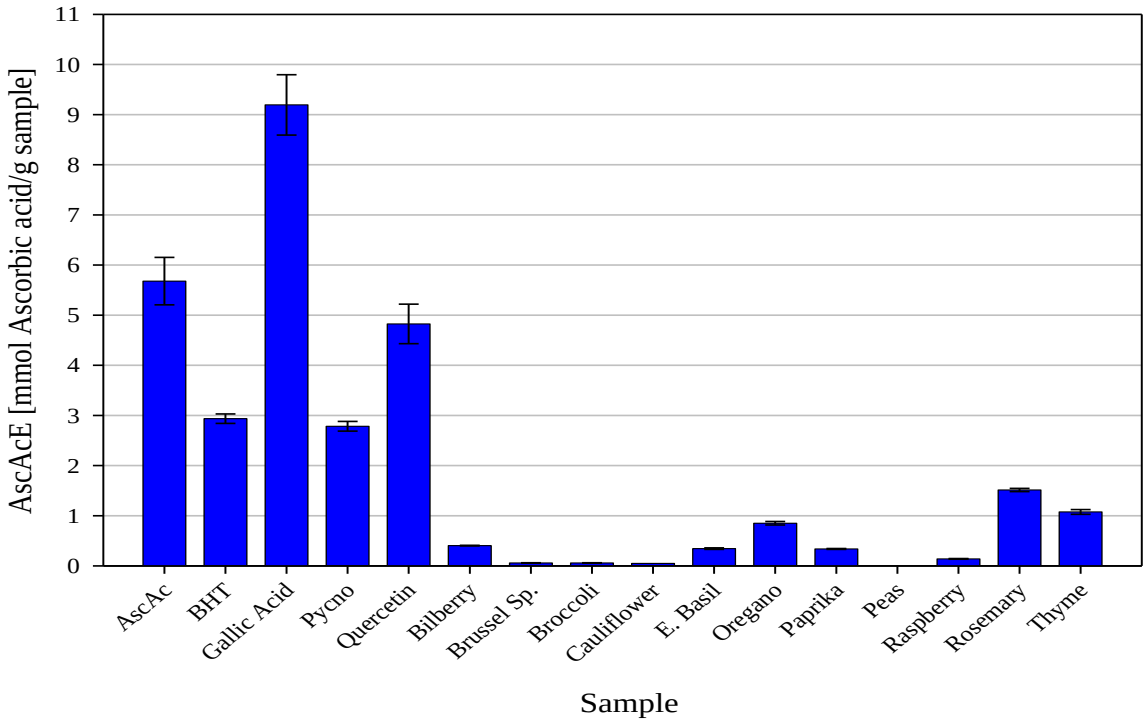

Figure 7. The reducing potential of tested samples compared with pure substances. The reducing potential is presented as ascorbic acid equivalents (AscAcE). AscAc = ascorbic acid; BHT = butylated hydroxytoluene; Pycno = Pycnogenol.

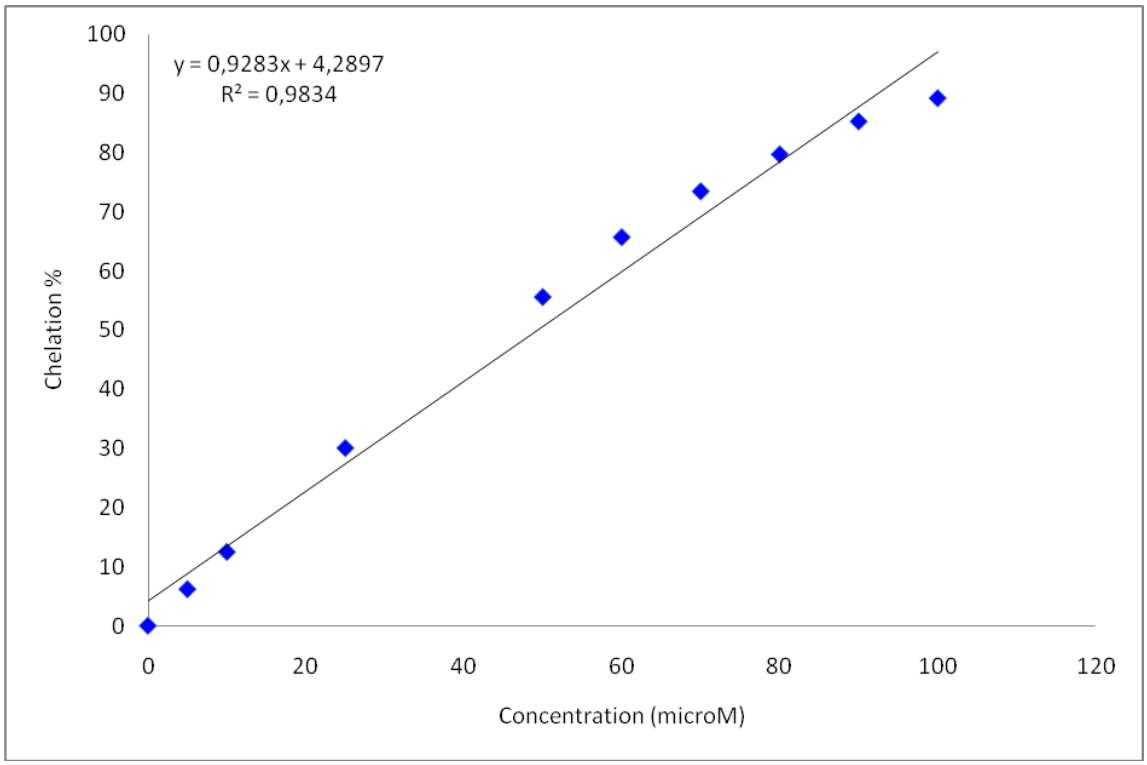

Figure 8. EDTA standard curve.

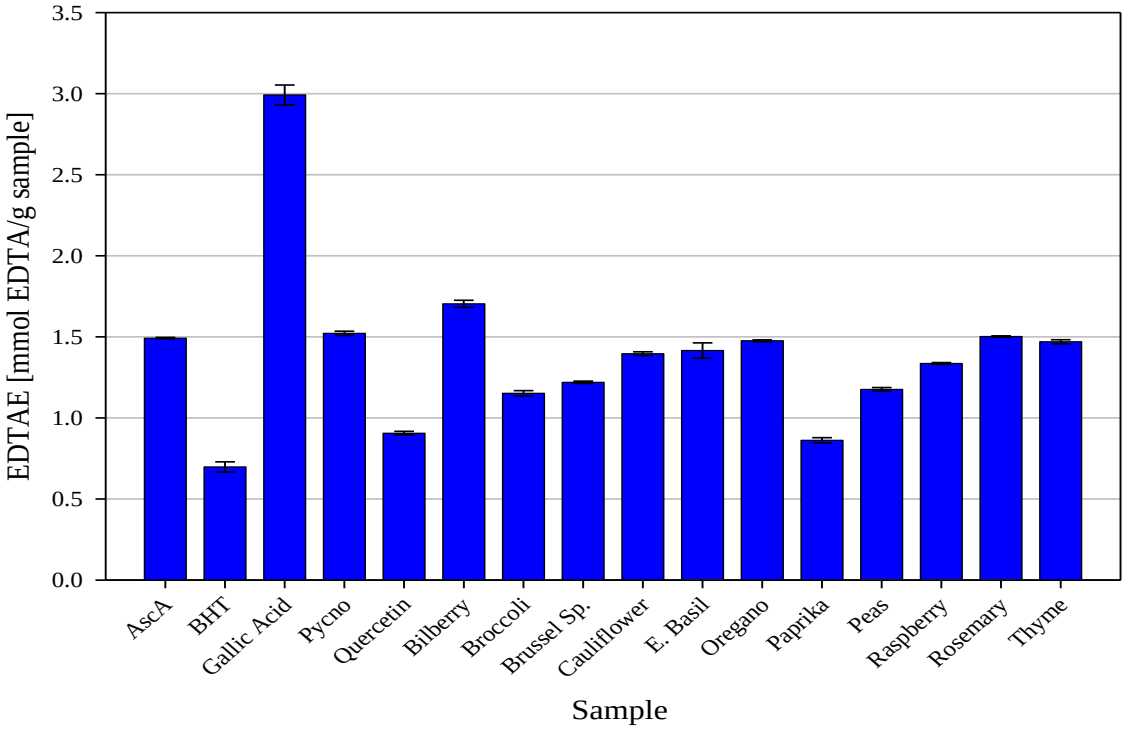

Figure 9. The ability of tested samples and pure antioxidant substances to chelate iron expressed as EDTA equivalents. AscA = ascorbic acid; BHT = butylated hydroxytoluene; Pycno = Pycnogenol

Interestingly, iron chelation potential of samples was approximately the same varying from 1.0 to 1.5 mmol EDTA / g sample with exception of paprika and bilberry. The value of paprika sample was the lowest among tested samples, while bilberry sample has proved to be even stronger iron chelator than ascorbic acid, BHT, Pycnogenol and quercetin. However, as mentioned previously (see 3.1. Mechanisms of action of antioxidants), the ability of a sample to chelate iron does not directly mean its ability to inhibit the oxidation.

8. DISCUSSION

Eleven samples of commonly used plant foods were tested for their antioxidant-related properties *in vitro*. Four simple assays were used for this purpose, and the results obtained were somewhat expected. To obtain more information on how different antioxidant activities of a sample are related to each other, correlation curves were

obtained (Appendix 5; Figures 10 and 11).

Total phenolic content of a sample do not necessarily correlate with its antioxidant capacity. A sample with low level of phenols may be relatively strong free radical scavenger as shown in this research (Figure 10) and in earlier one conducted by Wojdylo and colleagues (2007). However, some researchers were able to obtain good linear correlation between these two features in their studies. It was suggested that too small number of samples as well as too small difference between the highest and the lowest value of total phenolic content may provide a possible explanation for the poor correlative relationship (Cai et al., 2004; Shan et al., 2005). The correlation can also be influenced by extraction procedures, assay methods and solvents (Shan et al., 2005; Wojdylo et al., 2007; Sariburun et al., 2010). One possible reason for the absence of correlation between total phenol content and antioxidant activity could lie in the nature of the compounds responsible for the antioxidant-related activity of the sample (Shan et al., 2005; Wojdylo et al., 2007). It can also explain the fact that correlation may occur within a single plant family or group, but there is no correlation between different groups of plants, for example between fruits and vegetables (Wu et al., 2004; Wojdylo et al., 2007).

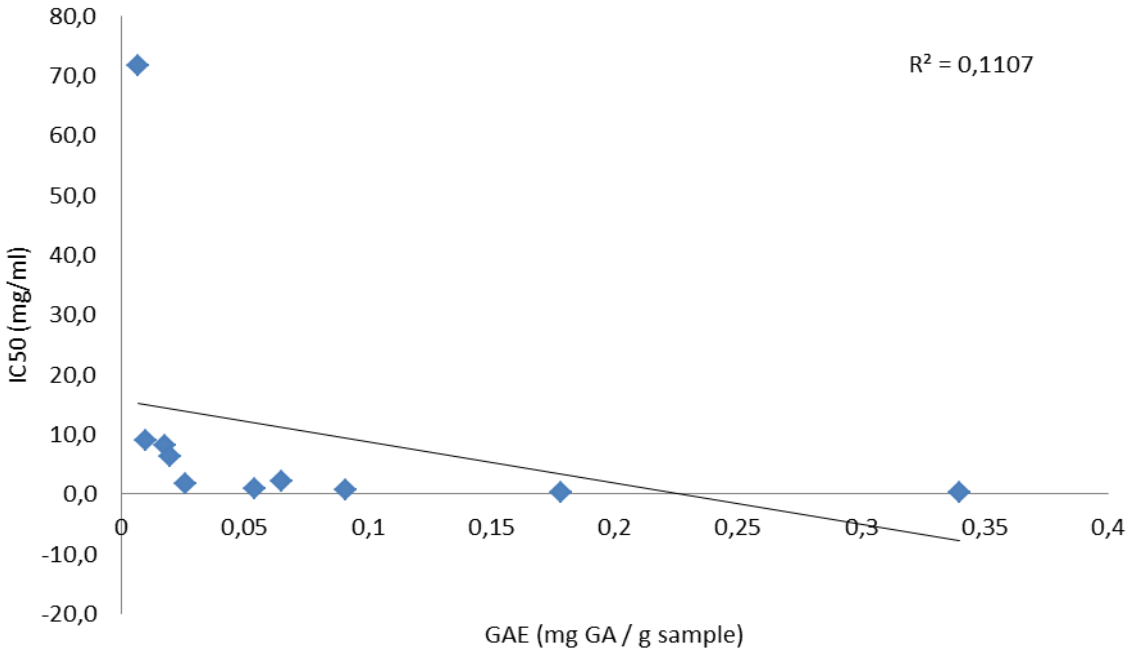

Figure 10. There was no correlation between total phenols and free radical scavenging capacity (IC_{50} values) of tested samples. GAE = gallic acid equivalents.

As can be seen also from other correlation curves (see Appendix V), the results obtained from different assays in this research are not consistent with each other. It means that the mechanisms behind potential antioxidant activity of tested samples are diverse, and thus testing samples of plant-origin with only one single method would not give a proper picture on the actual *in vitro* antioxidant capacities. Good correlative relationship occurs between total phenols and iron reduction, which can be explained by similar mechanisms (Figure 11). In both assays it is the reducing capacity of phenolic compounds which is actually measured.

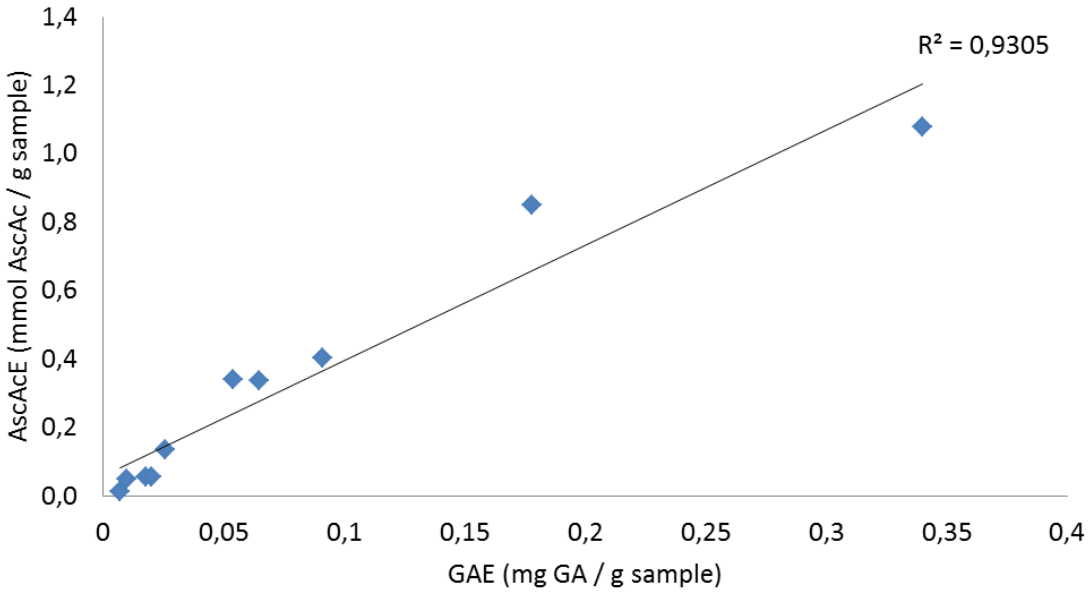

Figure 11. Correlation between total phenols and reducing power of tested samples. AscAcE = ascorbic acid; GAE = gallic acid equivalents

The research on antioxidant-related properties shows that there can be significant differences between antioxidant activities of different plant foods. Herbs seem to have higher total phenolic content and to be more active in scavenging free radicals and reducing iron compared to other tested samples. Berries, especially bilberry, are also more active as a potential source of antioxidants than vegetables. The data obtained in this research is consistent with earlier studies revealing better antioxidant capacity of *Vaccinium* species compared with fruits and vegetables (Cao et al., 1996; Prior et al., 1998).

However, my results do not necessarily agree with all previous studies. The problem with comparing results from different investigations lies in different techniques, solvents and extraction methods used. The choice of solvent for extraction of samples may have a profound effect on observed antioxidant content and antioxidant capacity (Szeto et al., 2002). Issues to take into account when measuring activities of plant extracts are, amongst others, pH, lipophilicity of solvents and processing temperature. As chopping, shredding and puréeing of plant samples may result in rapid and significant loss of antioxidant content, the risk of measurement inaccuracy should also be considered (Szeto et al., 2002). In addition, individual assays cannot reflect the real antioxidant activity of a sample, and thus the same sample tested with two different methods can give even totally different results (Huang et al., 2005).

On the basis of observed activities, it can be assumed that herbs and berries may be the main target for the research of pharmaceutically important compounds possessing antioxidant activity. In daily diet, however, vegetables (including roots and tubers) and fruits are likely to be the best sources of antioxidants rather than herbs and berries due to relatively lesser consumption of the latter. However, this may not be truth for everyone. As estimated from the Household Budget Survey, in Norway fruits and berries are a greater source of antioxidants than vegetables and roots (Halvorsen et al., 2002).

It still remains to be considered what doses of plant foods need to be consumed daily to provide the organism with efficient amounts of antioxidants. Generally, the health authorities recommend a dose of at least 500 grams of vegetables, fruits and berries a day. However, this recommendation does not take into account the amounts of antioxidants in each particular plant group or species. As antioxidant content may vary significantly even in two different specimens of the same plant species (for example, in two individual apples), it may be quite difficult to estimate the precise intake of antioxidants from diet. Antioxidant content variations may take place due to distinct growth, transportation, storage and processing conditions. Thereby, it is possible that two persons having identical diet may receive different amounts of antioxidants. Nonetheless, daily consumption of plant foods may exert beneficial impacts other than

those related to antioxidant activity.

Further research is needed to provide information on the activity of antioxidants and plant foods *in vivo*. Antioxidant defence is a result of the cooperative action of many different compounds. Thus being effective *in vitro,* a single compound (or sample containing this compound) may not be effective enough *in vivo* when used alone. This problem may arise particularly in drug development when using antioxidant-related properties as a mechanism of action. The same counts for consuming antioxidants as dietary supplements: a single vitamin or a complex of vitamins may not be as beneficial as healthy diet containing all those vitamins and many other, still partly unknown compounds. In addition, one should not think that an antioxidant compound is only good and safe. For example, excessive amounts of vitamin E may be detrimental to smokers, while the intake of antioxidants during chemotherapy may interfere with the efficacy of treatment (Weinberg et al., 2001; Laurent et al., 2005). It is possible that the consumption of commercial antioxidants far exceeding the recommended doses may sooner or later cause harm even in relatively healthy organism by disrupting its normal functions or acting as pro-oxidants.

Although epidemiological studies have revealed the connection between the consumption of plant foods and the decreased risk of oxidation-related diseases such as cancer and cardiovascular diseases, it is still unclear how profound a part antioxidants obtained from foods play in prevention and treatment of such diseases. It is possible that vegetables, fruits, berries, herbs and spices contain other components that can exhibit mechanisms different from those of antioxidants. Such components may also act in synergy with antioxidants and other compounds.

The identification, isolation and testing of active compounds could provide important information on the mechanisms of antioxidant activity of given samples as well as on the possible pro-oxidative activity. Pro-oxidative activity is based on the fact that any substance that can donate protons or electrons become a reactive species itself and thus can, in some cases, damage cells. Pharmaceutical development may benefit from both the antioxidant and pro-oxidant activity of a sample. While antioxidants could be used

in protecting healthy tissues from oxidative stress and recovering the free-radical damage, pro-oxidants could act as chemotherapeutic agents to kill cancer cells. Thus the question to be answered in the future researches is if we can use plant foods not only as healthy constituent of our diet, but as a potential source of important medicines.

9. CONCLUSIONS

On the basis of literature review, the association of oxidative stress with various conditions and diseases seems to be obvious, although the significance of this association still often remains unclear. The research in this field is invaluable in providing information on possible mechanisms of diseases and new potential ways of prevention and treatment of ones. Antioxidants and antioxidant-rich foods are thought to play an important part in promoting human health, and they are often suggested to be a beneficial addition to the conventional treatments of various conditions. Unfortunately, in vast majority of cases scientific evidence of *in vivo* antioxidant action remains insufficient. In addition, some antioxidant-related compounds may in certain occasions cause even harmful effects in the body.

Antioxidant-rich foods include various vegetables, fruits, berries and herbs, and are recommended for daily use. Different culinary plant groups exert different antioxidant-related activities, but there are also great capacity variations within each group. In this research, eleven samples of commonly used culinary plants were tested using four simple and widely used *in vitro* assays. Rosemary, thyme, oregano and bilberry are proved to possess the best free radical scavenging and reducing capacity, and vegetables, in particular peas, were the weakest samples in terms of antioxidant-related properties. Nonetheless, all samples were relatively good in chelating ferrous ions.

The results of this research cannot be directly applied *in vivo*. However, they may prove valuable in choosing the direction for further investigations. Because *in vitro* inactive compound is unlikely to be effective *in vivo*, it is worth focusing on the samples showing the highest antioxidant capacity. On the basis of this thesis, I would

particularly recommend herbs and berries for closer examination, which may prove useful at least for pharmaceutical and food industry.

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APPENDIX 1. The determination of total phenols data. Absorbance was measured for 0.5 mg/ml and 1 mg/ml samples in five replicates and then mean values and standard deviation were calculated. The mean absorbance values of 1 mg/ml samples were used to calculate gallic acid equivalents. See section 6.5.1. for experimental details.

	Bilberry			***Broccoli***			***Brussel sprouts***	
	0,5 mg/ml	*1 mg/ml*		*0,5 mg/ml*	*1 mg/ml*		*0,5 mg/ml*	*1 mg/ml*
	0,186	0,483		0,022	0,079		0,037	0,036*
	0,189	0,463		0,024	0,072		0,036	0,083
	0,203	0,472		0,024	0,074		0,038	0,083
	0,216	0,495		0,024	0,073		0,037	0,083
	0,225	0,500		0,026	0,079		0,038	0,085
Mean	0,204	0,483		0,024	0,075		0,037	0,084
SD	0,017	0,015		0,001	0,003		0,001	0,001
							* wrong pipetting? (not incl.)	
	Cauliflower			***Egyptian basil***			***Oregano***	
	0,5 mg/ml	*1 mg/ml*		*0,5 mg/ml*	*1 mg/ml*		*0,5 mg/ml*	*1 mg/ml*
	0,011	0,031		0,156	0,272		0,511	0,955
	0,016	0,031		0,155	0,288		0,512	0,974
	0,012	0,033		0,154	0,284		0,503	0,959
	0,012	0,031		0,156	0,262		0,493	0,991
	0,020	0,032		0,150	0,274		0,485	0,954
Mean	0,014	0,032		0,154	0,276		0,501	0,967
SD	0,004	0,001		0,002	0,010		0,012	0,016
	Paprika			***Peas***			***Raspberry***	
	0,5 mg/ml	*1 mg/ml*		*0,5 mg/ml*	*1 mg/ml*		*0,5 mg/ml*	*1 mg/ml*
	0,175	0,338		0,006	0,015		0,038	0,115
	0,179	0,334		0,005	0,015		0,040	0,118
	0,172	0,330		0,005	0,016		0,040	0,126
	0,185	0,336		0,005	0,014		0,041	0,118
	0,176	0,341		0,005	0,014		0,038	0,116
Mean	0,177	0,336		0,005	0,015		0,039	0,119
SD	0,005	0,004		0,000	0,001		0,001	0,004
	Thyme							
	0,5 mg/ml	*1 mg/ml*						
	0,752	1,870						
	0,690	1,911						
	0,719	1,827						
	0,711	1,891						
	0,710	1,868						
Mean	0,716	1,873						
SD	0,023	0,031						

APPENDIX 2. The DPPH free radical scavenging assay data. Inhibition percentage is calculated from absorbance. See section 6.5.2. for experimental details.

Bilberry

	Replicates								
mg/ml	**1**	**2**	**3**	**4**	**5**	**Mean**	**SD**	**SEM**	**95% CI**
0	0,000 %	0,000 %	0,000 %	0,000 %	0,000 %	0,000 %	0,000	0,000	0,000
0,001	2,439 %	-0,890 %	-0,888 %	-2,246 %	-0,293 %	-0,376 %	0,017	0,008	0,015
0,01	2,869 %	-0,445 %	-0,444 %	-1,048 %	0,440 %	0,275 %	0,015	0,007	0,014
0,1	9,182 %	6,825 %	6,361 %	5,689 %	8,211 %	7,254 %	0,014	0,006	0,012
0,25	19,225 %	15,134 %	15,976 %	15,569 %	17,302 %	16,641 %	0,017	0,007	0,015
0,5	36,585 %	34,570 %	35,947 %	34,581 %	34,897 %	35,316 %	0,009	0,004	0,008
1	67,145 %	66,469 %	67,456 %	67,814 %	68,328 %	67,442 %	0,007	0,003	0,006
2,5	88,235 %	87,982 %	87,722 %	87,575 %	88,123 %	87,927 %	0,003	0,001	0,002
5	82,927 %	82,344 %	82,249 %	81,587 %	83,871 %	82,595 %	0,009	0,004	0,008

Broccoli

mg/ml	**1**	**2**	**3**	**4**	**5**	**Mean**	**SD**	**SEM**	**95 % CI**
0	0,000 %	0,000 %	0,000 %	0,000 %	0,000 %	0,000 %	0,000	0,000	0,000
0,1	-2,339 %	0,714 %	-1,153 %	0,290 %	0,291 %	-0,439 %	0,013	0,006	0,011
0,5	0,146 %	3,429 %	2,450 %	3,043 %	2,907 %	2,395 %	0,013	0,006	0,011
1	3,509 %	6,857 %	7,349 %	6,812 %	5,814 %	6,068 %	0,015	0,007	0,013
2,5	12,281 %	15,429 %	17,147 %	16,232 %	15,262 %	15,270 %	0,018	0,008	0,016
5	27,485 %	30,286 %	31,844 %	31,304 %	30,087 %	30,201 %	0,017	0,008	0,015
10	49,269 %	55,286 %	55,620 %	57,536 %	55,233 %	54,589 %	0,031	0,014	0,027
20	89,766 %	91,857 %	91,931 %	91,449 %	91,860 %	91,373 %	0,009	0,004	0,008
50	90,936 %	91,000 %	91,066 %	91,159 %	91,134 %	91,059 %	0,001	0,000	0,001

Brussels sprouts

mg/ml	**1**	**2**	**3**	**4**	**5**	**Mean**	**SD**	**SEM**	**95 % CI**
0	0,000 %	0,000 %	0,000 %	0,000 %	0,000 %	0,000 %	0,000	0,000	0,000
0,1	-0,905 %	-1,240 %	0,916 %	1,064 %	0,762 %	0,119 %	0,011	0,005	0,010
0,5	2,262 %	2,016 %	4,427 %	4,407 %	3,963 %	3,415 %	0,012	0,005	0,010
1	6,335 %	6,512 %	7,939 %	8,359 %	7,470 %	7,323 %	0,009	0,004	0,008
2,5	17,798 %	18,450 %	19,542 %	20,365 %	19,665 %	19,164 %	0,010	0,005	0,009
5	33,032 %	33,643 %	34,656 %	35,258 %	35,061 %	34,330 %	0,010	0,004	0,008
10	69,382 %	70,543 %	71,756 %	69,301 %	70,274 %	70,251 %	0,010	0,004	0,009
25	90,799 %	90,698 %	90,840 %	90,881 %	90,854 %	90,814 %	0,001	0,000	0,001
50	89,744 %	89,767 %	90,076 %	90,274 %	90,091 %	89,990 %	0,002	0,001	0,002

Cauliflower

mg/ml	**1**	**2**	**3**	**4**	**5**	**Mean**	**SD**	**SEM**	**95 % CI**
0	0,000 %	0,000 %	0,000 %	0,000 %	0,000 %	0,000 %	0,000	0,000	0,000
0,01	-1,029 %	0,146 %	-0,588 %	1,744 %	-0,146 %	0,025 %	0,011	0,005	0,009
0,1	-1,176 %	1,023 %	0,441 %	1,163 %	0,439 %	0,378 %	0,009	0,004	0,008
0,5	1,324 %	4,094 %	1,471 %	2,907 %	1,903 %	2,340 %	0,012	0,005	0,010
1	4,559 %	5,848 %	6,029 %	5,669 %	5,271 %	5,475 %	0,006	0,003	0,005
2,5	12,206 %	13,304 %	12,353 %	12,645 %	12,006 %	12,503 %	0,005	0,002	0,004
5	27,059 %	27,047 %	26,618 %	27,616 %	26,940 %	27,056 %	0,004	0,002	0,003
10	52,206 %	53,363 %	54,412 %	53,779 %	51,977 %	53,147 %	0,010	0,005	0,009
25	92,353 %	92,251 %	92,206 %	92,442 %	92,387 %	92,328 %	0,001	0,000	0,001
50	92,059 %	92,105 %	91,912 %	92,151 %	92,094 %	92,064 %	0,001	0,000	0,001

APPENDIX 2 (continued)

Egyptian basil

mg/ml	1	2	3	4	5	Mean	SD	SEM	95 % CI
0	0,000 %	0,000 %	0,000 %	0,000 %	0,000 %	0,000 %	0,000	0,000	0,000
0,01	-4,141 %	0,767 %	1,074 %	1,072 %	1,991 %	0,152 %	0,024	0,011	0,021
0,1	4,601 %	9,509 %	9,816 %	9,954 %	8,576 %	8,491 %	0,022	0,010	0,020
0,25	7,362 %	12,270 %	13,497 %	13,783 %	13,936 %	12,169 %	0,028	0,012	0,024
0,5	22,546 %	27,607 %	27,607 %	29,403 %	27,718 %	26,976 %	0,026	0,012	0,023
1	46,472 %	48,006 %	53,834 %	52,986 %	53,139 %	50,888 %	0,034	0,015	0,030
2,5	92,485 %	91,411 %	91,258 %	91,424 %	91,118 %	91,539 %	0,005	0,002	0,005
5	92,178 %	91,104 %	91,104 %	91,118 %	91,118 %	91,324 %	0,005	0,002	0,004

Oregano

mg/ml	1	2	3	4	5	Mean	SD	SEM	95 % CI
0	0,000 %	0,000 %	0,000 %	0,000 %	0,000 %	0,000 %	0,000	0,000	0,000
0,001	-0,650 %	0,213 %	-0,323 %	0,484 %	1,768 %	0,298 %	0,009	0,004	0,008
0,0125	0,976 %	1,667 %	1,616 %	1,935 %	3,215 %	1,882 %	0,008	0,004	0,007
0,05	5,691 %	6,511 %	6,139 %	6,774 %	7,074 %	6,438 %	0,005	0,002	0,005
0,125	16,585 %	17,006 %	17,286 %	17,097 %	18,489 %	17,293 %	0,007	0,003	0,006
0,25	31,545 %	34,767 %	34,733 %	34,839 %	35,048 %	34,186 %	0,015	0,007	0,013
0,5	62,439 %	61,248 %	61,874 %	62,419 %	62,058 %	62,008 %	0,005	0,002	0,004
1	90,081 %	90,312 %	90,307 %	90,645 %	90,675 %	90,404 %	0,003	0,001	0,002
2,5	90,081 %	90,151 %	90,145 %	90,484 %	90,514 %	90,275 %	0,002	0,001	0,002

Paprika

mg/ml	1	2	3	4	5	Mean	SD	SEM	95 % CI
0	0,000 %	0,000 %	0,000 %	0,000 %	0,000 %	0,000 %	0,000	0,000	0,000
0,01	-4,290 %	-0,144 %	0,430 %	-0,308 %	-0,153 %	-0,893 %	0,019	0,009	0,017
0,05	-2,367 %	1,153 %	0,143 %	1,231 %	1,223 %	0,277 %	0,015	0,007	0,014
0,1	-1,479 %	1,297 %	7,593 %	1,846 %	1,223 %	2,096 %	0,033	0,015	0,029
0,5	6,213 %	9,366 %	15,759 %	9,846 %	10,398 %	10,316 %	0,034	0,015	0,030
1	16,124 %	17,723 %	25,072 %	19,846 %	19,725 %	19,698 %	0,034	0,015	0,030
5	69,231 %	70,317 %	75,645 %	74,615 %	72,783 %	72,518 %	0,027	0,012	0,024
10	87,278 %	87,464 %	88,395 %	86,769 %	87,462 %	87,474 %	0,006	0,003	0,005
20	84,615 %	85,014 %	85,673 %	84,615 %	84,404 %	84,864 %	0,005	0,002	0,004

Peas

mg/ml	1	2	3	4	5	Mean	SD	SEM	95 % CI
0	0,000 %	0,000 %	0,000 %	0,000 %	0,000 %	0,000 %	0,000	0,000	0,000
1	-2,624 %	0,708 %	1,416 %	0,431 %	0,000 %	-0,014 %	0,015	0,007	0,014
2,5	-1,020 %	2,408 %	3,258 %	3,879 %	1,873 %	2,080 %	0,019	0,008	0,017
5	1,895 %	5,099 %	6,091 %	5,747 %	4,755 %	4,717 %	0,017	0,007	0,015
10	8,601 %	11,756 %	13,598 %	12,500 %	11,527 %	11,596 %	0,019	0,008	0,016
20	21,574 %	23,513 %	24,079 %	25,144 %	24,207 %	23,704 %	0,013	0,006	0,012
50	55,394 %	57,365 %	56,374 %	57,184 %	57,061 %	56,675 %	0,008	0,004	0,007
75	77,697 %	77,620 %	78,754 %	82,328 %	82,133 %	79,706 %	0,023	0,011	0,021
100	91,545 %	91,501 %	92,068 %	90,948 %	92,075 %	91,628 %	0,005	0,002	0,004

APPENDIX 2 (continued)

Raspberry

mg/ml	1	2	3	4	5	Mean	SD	SEM	95 % CI
0	0,000 %	0,000 %	0,000 %	0,000 %	0,000 %	0,000 %	0,000	0,000	0,000
0,01	-2,465 %	0,751 %	0,152 %	0,304 %	0,304 %	-0,191 %	0,013	0,006	0,011
0,1	0,308 %	3,754 %	4,255 %	3,343 %	3,349 %	3,002 %	0,016	0,007	0,014
0,5	9,245 %	11,562 %	11,550 %	12,614 %	12,938 %	11,582 %	0,014	0,006	0,013
1	23,112 %	25,526 %	26,292 %	25,228 %	26,180 %	25,267 %	0,013	0,006	0,011
2,5	65,177 %	65,916 %	66,261 %	64,742 %	68,037 %	66,027 %	0,013	0,006	0,011
5	90,447 %	90,090 %	90,426 %	90,426 %	90,259 %	90,329 %	0,002	0,001	0,001
10	89,831 %	90,090 %	90,122 %	89,970 %	89,954 %	89,993 %	0,001	0,001	0,001

Rosemary

mg/ml	1	2	3	4	5	Mean	SD	SEM	95 % CI
0	0,000 %	0,000 %	0,000 %	0,000 %	0,000 %	0,000 %	0,000	0,000	0,000
0,001	3,436 %	1,449 %	1,268 %	1,630 %	2,712 %	2,099 %	0,009	0,004	0,008
0,01	3,978 %	5,435 %	4,891 %	5,435 %	6,148 %	5,177 %	0,008	0,004	0,007
0,05	17,360 %	17,754 %	19,022 %	19,384 %	19,349 %	18,574 %	0,009	0,004	0,008
0,125	34,358 %	34,601 %	34,601 %	36,413 %	38,517 %	35,698 %	0,018	0,008	0,016
0,25	62,568 %	64,674 %	64,855 %	67,029 %	72,875 %	66,400 %	0,039	0,018	0,035
0,5	87,161 %	86,775 %	87,319 %	87,681 %	87,703 %	87,328 %	0,004	0,002	0,003
1	87,161 %	87,319 %	87,319 %	87,862 %	87,523 %	87,437 %	0,003	0,001	0,002

Thyme

mg/ml	1	2	3	4	5	Mean	SD	SEM	95 % CI
0	0,000 %	0,000 %	0,000 %	0,000 %	0,000 %	0,000 %	0,000	0,000	0,000
0,005	-0,167 %	1,967 %	2,128 %	2,145 %	1,495 %	1,514 %	0,010	0,004	0,009
0,0125	0,502 %	3,607 %	4,255 %	4,455 %	3,322 %	3,228 %	0,016	0,007	0,014
0,025	2,341 %	5,574 %	6,056 %	6,106 %	5,482 %	5,112 %	0,016	0,007	0,014
0,05	7,692 %	10,328 %	10,311 %	10,891 %	11,130 %	10,070 %	0,014	0,006	0,012
0,125	21,739 %	23,279 %	24,386 %	24,917 %	22,093 %	23,283 %	0,014	0,006	0,012
0,25	48,161 %	51,803 %	51,718 %	52,145 %	54,817 %	51,729 %	0,024	0,011	0,021
0,5	87,960 %	89,344 %	89,362 %	89,439 %	89,867 %	89,194 %	0,007	0,003	0,006
1	89,632 %	90,000 %	90,016 %	89,934 %	89,867 %	89,890 %	0,002	0,001	0,001

APPENDIX 3. Reducing power activity assay.

Bilberry

mg/ml	1	2	3	4	5	Mean	SD	SEM	95 % CI
0	-0,019	-0,015	-0,025	-0,019	-0,019	-0,019	0,004	0,002	0,003
0,1	0,074	0,090	0,081	0,081	0,082	0,082	0,006	0,003	0,005
0,25	0,202	0,212	0,194	0,198	0,195	0,200	0,007	0,003	0,006
0,5	0,432	0,413	0,411	0,405	0,414	0,415	0,010	0,005	0,009
1	0,830	0,852	0,787	0,814	0,820	0,821	0,024	0,011	0,021
2,5	1,792	1,784	1,713	1,708	1,771	1,754	0,040	0,018	0,035
5	3,066	3,075	3,119	3,078	3,140	3,096	0,032	0,014	0,028

Broccoli

mg/ml	1	2	3	4	5	Mean	SD	SEM	95 % CI
0	-0,026	-0,027	-0,027	-0,025	-0,029	-0,027	0,001	0,001	0,001
0,5	0,008	0,007	0,013	0,009	0,010	0,009	0,002	0,001	0,002
1	0,082	0,080	0,086	0,090	0,082	0,084	0,004	0,002	0,004
2,5	0,227	0,231	0,237	0,232	0,234	0,232	0,004	0,002	0,003
5	0,476	0,478	0,496	0,483	0,467	0,480	0,011	0,005	0,009
10	0,898	0,917	0,908	0,850	0,894	0,893	0,026	0,012	0,023
20	1,746	1,701	1,627	1,708	1,713	1,699	0,044	0,020	0,038

Brussels sprouts

mg/ml	1	2	3	4	5	Mean	SD	SEM	95 % CI
0	-0,018	-0,020	-0,021	-0,020	-0,020	-0,020	0,001	0,000	0,001
0,5	0,036	0,046	0,040	0,049	0,049	0,044	0,006	0,003	0,005
1	0,112	0,111	0,107	0,113	0,112	0,111	0,002	0,001	0,002
2,5	0,273	0,273	0,269	0,268	0,283	0,273	0,006	0,003	0,005
5	0,484	0,475	0,452	0,472	0,487	0,474	0,014	0,006	0,012
10	0,989	1,046	1,039	1,033	1,012	1,024	0,023	0,010	0,020
*** 25**	1,906	1,896	1,936	1,891	1,918	1,909	0,018	0,008	0,016
* slight precipitation									

Cauliflower

mg/ml	1	2	3	4	5	Mean	SD	SEM	95 % CI
0	-0,020	-0,020	-0,019	-0,019	-0,020	-0,020	0,001	0,000	0,000
0,5	0,034	0,031	0,051	0,035	0,035	0,037	0,008	0,004	0,007
1	0,095	0,092	0,106	0,095	0,096	0,097	0,005	0,002	0,005
2,5	0,216	0,210	0,217	0,206	0,215	0,213	0,005	0,002	0,004
5	0,460	0,448	0,456	0,454	0,448	0,453	0,005	0,002	0,005
10	0,892	0,857	0,874	0,871	0,842	0,867	0,019	0,008	0,016
25	1,902	1,837	2,006	1,960	1,724	1,886	0,110	0,049	0,097

Egyptian basil

mg/ml	1	2	3	4	5	Mean	SD	SEM	95 % CI
0	-0,024	-0,023	-0,022	-0,022	-0,022	-0,023	0,001	0,000	0,001
0,075	0,029	0,030	0,028	0,031	0,030	0,030	0,001	0,001	0,001
0,125	0,063	0,066	0,071	0,075	0,071	0,069	0,005	0,002	0,004
0,25	0,163	0,162	0,163	0,167	0,164	0,164	0,002	0,001	0,002
0,5	0,321	0,341	0,317	0,348	0,359	0,337	0,018	0,008	0,016
1	0,580	0,543	0,532	0,573	0,593	0,564	0,026	0,011	0,023
2,5	1,391	1,289	1,287	1,260	1,304	1,306	0,050	0,022	0,044

APPENDIX 3 (continued)

Oregano

mg/ml	1	2	3	4	5	Mean	SD	SEM	95 % CI
0	-0,023	-0,022	-0,022	-0,022	-0,022	-0,022	0,000	0,000	0,000
0,025	0,007	0,001	0,013	0,013	0,010	0,009	0,005	0,002	0,004
0,05	0,057	0,052	0,062	0,055	0,062	0,058	0,004	0,002	0,004
0,075	0,098	0,096	0,100	0,102	0,105	0,100	0,003	0,002	0,003
0,125	0,183	0,183	0,192	0,191	0,188	0,187	0,004	0,002	0,004
0,25	0,365	0,374	0,378	0,351	0,366	0,367	0,010	0,005	0,009
0,5	0,600	0,604	0,673	0,685	0,682	0,649	0,043	0,019	0,038
1	1,310	1,327	1,190	1,324	1,306	1,291	0,057	0,026	0,050

Paprika

mg/ml	1	2	3	4	5	Mean	SD	SEM	95 % CI
0	-0,023	-0,023	-0,025	-0,025	-0,024	-0,024	0,001	0,000	0,001
0,1	0,019	0,003	0,024	0,019	0,017	0,016	0,008	0,004	0,007
0,25	0,104	0,101	0,113	0,109	0,106	0,107	0,005	0,002	0,004
0,5	0,254	0,259	0,251	0,257	0,256	0,255	0,003	0,001	0,003
1	0,535	0,530	0,524	0,546	0,528	0,533	0,008	0,004	0,007
2,5	1,238	1,224	1,275	1,303	1,260	1,260	0,031	0,014	0,027

Peas

mg/ml	1	2	3	4	5	Mean	SD	SEM	95 % CI
0	-0,024	-0,024	-0,027	-0,083	-0,015	-0,035	0,027	0,012	0,024
2,5	0,024	0,030	0,021	-0,034	0,029	0,014	0,027	0,012	0,024
5	0,088	0,088	0,085	0,034	0,086	0,076	0,024	0,011	0,021
10	0,202	0,219	0,204	0,145	0,188	0,192	0,028	0,013	0,025
20	0,393	0,445	0,416	0,354	0,384	0,398	0,034	0,015	0,030
50	1,056	1,084	1,057	0,985	0,971	1,031	0,050	0,022	0,043
75	1,510	1,433	1,407	1,443	1,493	1,457	0,043	0,019	0,038

Raspberry

mg/ml	1	2	3	4	5	Mean	SD	SEM	95 % CI
0	-0,021	-0,021	-0,018	-0,020	-0,015	-0,019	0,003	0,001	0,002
0,25	0,046	0,055	0,049	0,052	0,048	0,050	0,004	0,002	0,003
0,5	0,100	0,114	0,115	0,104	0,105	0,108	0,007	0,003	0,006
1	0,240	0,246	0,250	0,246	0,235	0,243	0,006	0,003	0,005
2,5	0,673	0,659	0,696	0,653	0,662	0,669	0,017	0,008	0,015
5	1,275	1,183	1,254	1,234	0,908	1,171	0,151	0,067	0,132
*** 10**	2,167	2,103	2,068	2,049	1,952	2,068	0,079	0,035	0,069
* slight precipitation									

Rosemary

mg/ml	1	2	3	4	5	Mean	SD	SEM	95 % CI
0	-0,025	-0,025	-0,025	-0,026	-0,025	-0,025	0,000	0,000	0,000
0,01	-0,001	0,007	0,005	0,001	0,002	0,003	0,003	0,001	0,003
0,05	0,157	0,164	0,162	0,156	0,163	0,160	0,004	0,002	0,003
0,075	0,203	0,206	0,205	0,191	0,197	0,200	0,006	0,003	0,006
0,125	0,320	0,342	0,333	0,337	0,336	0,334	0,008	0,004	0,007
0,25	0,629	0,565	0,561	0,601	0,630	0,597	0,033	0,015	0,029
0,5	1,165	1,167	1,129	1,136	1,179	1,155	0,022	0,010	0,019

APPENDIX 3 (continued)

Thyme

mg/ml	1	2	3	4	5	Mean	SD	SEM	95 % CI
0	-0,025	-0,024	-0,024	-0,024	-0,023	-0,024	0,001	0,000	0,001
0,025	0,033	0,025	0,024	0,022	0,029	0,027	0,004	0,002	0,004
0,05	0,081	0,077	0,073	0,060	0,083	0,075	0,009	0,004	0,008
0,075	0,135	0,129	0,129	0,129	0,135	0,131	0,003	0,001	0,003
0,125	0,220	0,224	0,217	0,222	0,248	0,226	0,012	0,006	0,011
0,25	0,492	0,485	0,483	0,480	0,525	0,493	0,018	0,008	0,016
0,5	0,895	0,912	0,887	0,871	0,959	0,905	0,034	0,015	0,030
1	1,508	1,606	1,608	1,646	1,717	1,617	0,076	0,034	0,066

APPENDIX 4. Iron (II) chelation. EDTA equivalents were calculated from mean absorbance values of 1 mg/ml samples (when possible).

	Gallic acid		***BHT***			***Pycnogenol***	
	0,5 mg/ml		*0,5 mg/ml*	*1 mg/ml **		*0,5 mg/ml*	*1 mg/ml*
	1,387		0,305	2,018		1,420	1,410
	1,378		0,323	2,113		1,417	1,422
	1,372		0,329	2,155		1,412	1,410
	1,379		0,341	2,166		1,406	1,415
	1,442		0,337	2,160		1,428	1,435
Mean	1,392		0,327	2,122		1,417	1,418
SD	0,029		0,014	0,062		0,008	0,011
			* colour of samples was light				
			pink, much lighter than 0-sample				
			but it was opale, not transparent				

	Quercetin *			***Vitamin C***	
	0,5 mg/ml	*1 mg/ml*		*0,5 mg/ml*	*1 mg/ml*
	1,354	0,846		1,393	1,391
	1,360	0,857		1,389	1,390
	1,336	0,835		1,391	1,389
	1,352	0,843		1,405	1,390
	1,349	0,847		1,402	1,397
Mean	1,350	0,846		1,396	1,391
SD	0,009	0,008		0,007	0,003
	* samples became dark yellow				
	after the addition of Iron (II) sol.				
	in 1 mg/ml colour still dark yellow / brown				
	after the addition of ferrozine				

	Bilberry		***Broccoli***		***Brussel sprouts***	
	0,5 mg/ml	*1 mg/ml*	*0,5 mg/ml*	*1 mg/ml*	*0,5 mg/ml*	*1 mg/ml*
	1,459	1,562	1,192	1,052	1,241	1,148
	1,463	1,567	1,252	1,066	1,255	1,130
	1,468	1,599	1,143	1,082	1,239	1,139
	1,474	1,599	1,201	1,079	1,255	1,136
	1,479	1,602	1,191	1,087	1,248	1,139
Mean	1,469	1,586	1,196	1,073	1,248	1,138
SD	0,008	0,020	0,039	0,014	0,008	0,007

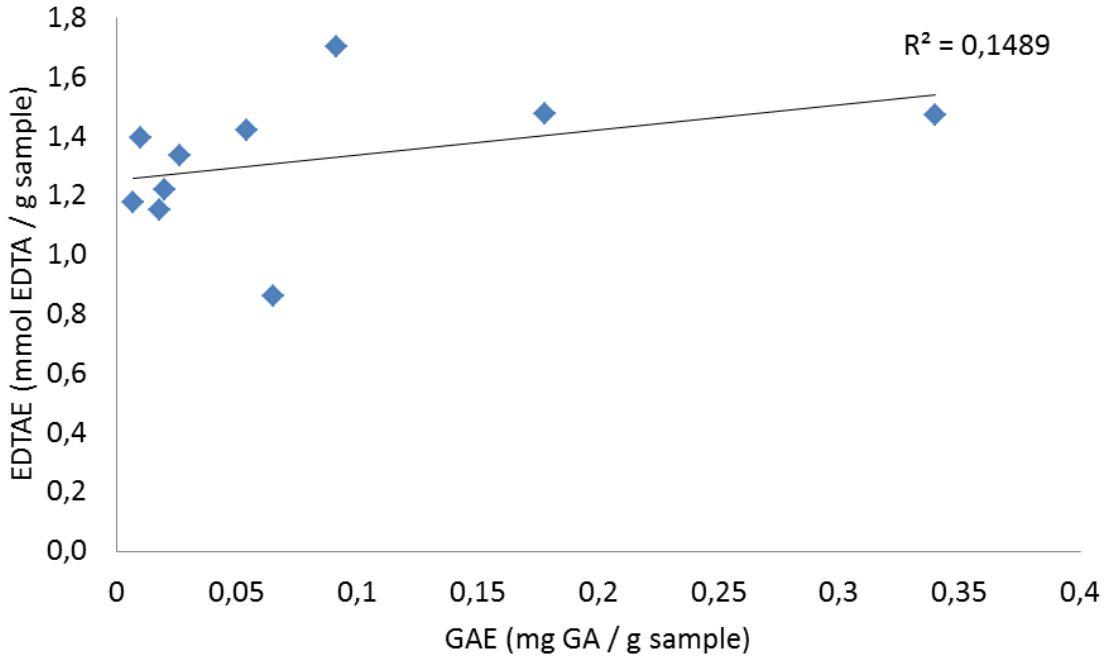
	Cauliflower		***Egyptian basil***		***Oregano***	
	0,5 mg/ml	*1 mg/ml*	*0,5 mg/ml*	*1 mg/ml*	*0,5 mg/ml*	*1 mg/ml*
	1,286	1,287	1,365	1,336	1,378	1,377
	1,298	1,303	1,350	1,335	1,377	1,369
	1,292	1,298	1,315	1,287	2,565*	2,539*
	1,281	1,301	1,357	1,270	1,382	1,376
	1,254	1,310	1,364	1,380	1,360	1,373
Mean	1,282	1,300	1,350	1,322	1,374	1,374
SD	0,017	0,008	0,021	0,044	0,010	0,004
					* Iron (II) solution added twice	
					(not included)	

APPENDIX 4 (continued)

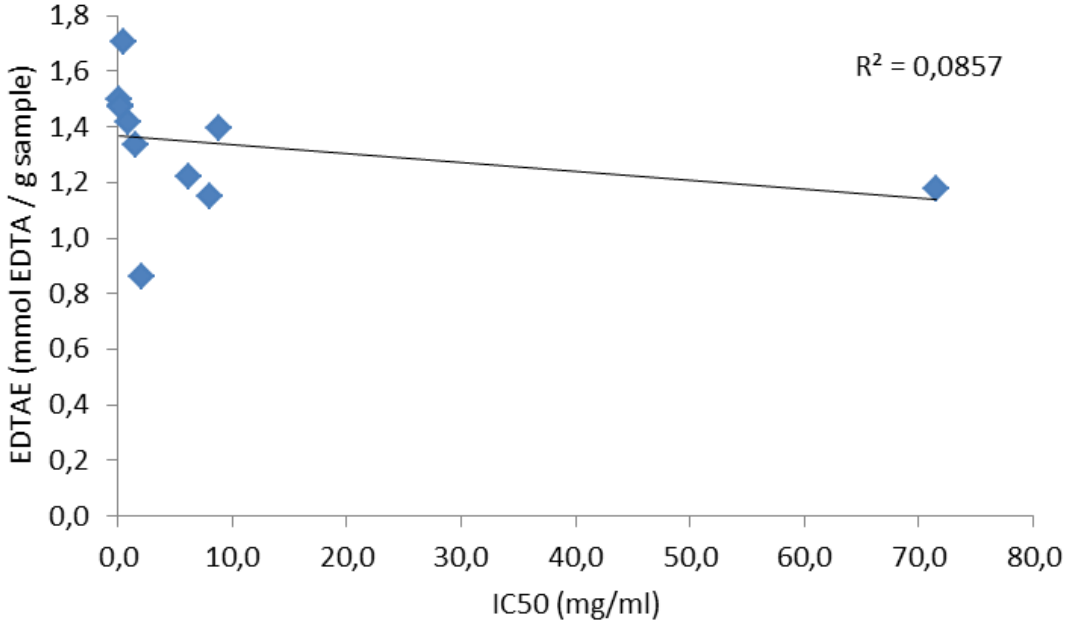
	Paprika			***Peas***			***Raspberry***	
	0,5 mg/ml	*1 mg/ml*		*0,5 mg/ml*	*1 mg/ml*		*0,5 mg/ml*	*1 mg/ml*
	1,147	0,803		1,251	1,108		1,270	1,237
	1,157	0,795		1,262	1,100		1,268	1,239
	1,154	0,803		1,236	1,094		1,279	1,245
	1,154	0,790		1,241	1,102		1,219	1,247
	1,163	0,830		1,258	1,078		1,265	1,246
Mean	1,155	0,804		1,250	1,096		1,260	1,243
SD	0,006	0,015		0,011	0,011		0,024	0,004
	Rosemary			***Thyme***				
	0,5 mg/ml	*1 mg/ml*		*0,5 mg/ml*	*1 mg/ml*			
	1,419	1,402		1,370	1,371			
	1,398	1,395		1,364	1,370			
	1,375	1,395		1,370	1,363			
	1,387	1,394		1,376	1,360			
	1,577*	1,396		1,361	1,388			
Mean	1,395	1,396		1,368	1,370			
SD	0,019	0,003		0,006	0,011			
	* sample amount incorrect							
	(not included)							

APPENDIX 5. Correlation curves.

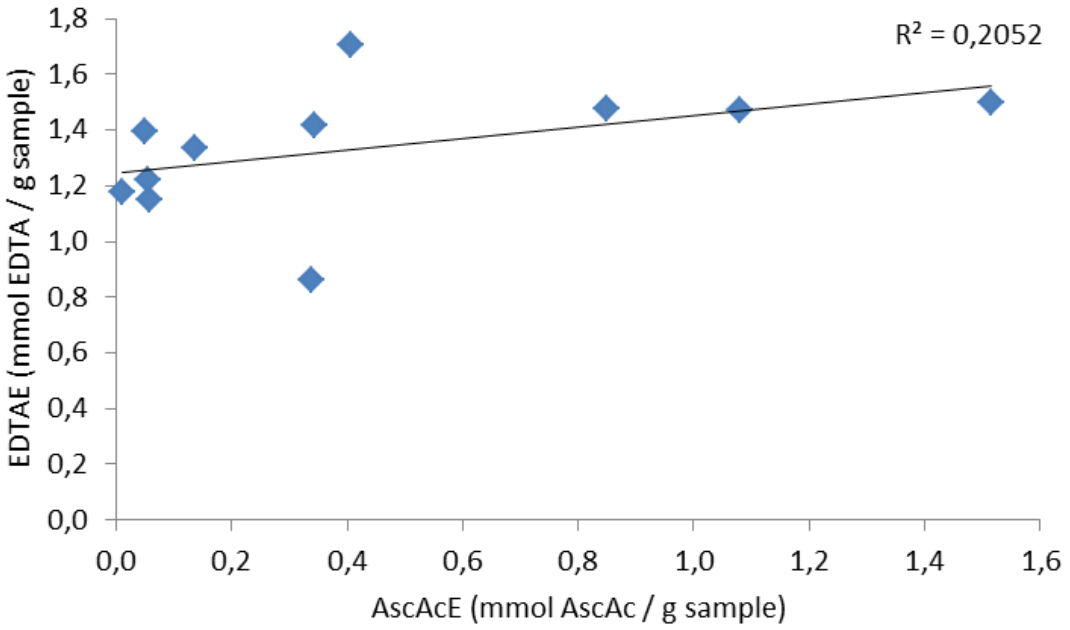
Correlation between total phenols and iron (II) chelation:



Correlation between iron chelation and free radical scavenging capacity:



Correlation between iron chelation and reducing power:



Correlation between free radical scavenging capacity and reducing power: